MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Dec. 2004, p. 639–668 Vol. 68, No. 4
1092-2172/04/$08.00+0 DOI: 10.1128/MMBR.68.4.639–668.2004

Control of rRNA Synthesis in *Escherichia coli*: a Systems Biology Approach†

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† We dedicate this review in gratitude to Gunther S. Stent, who set the field about the control of ribosome synthesis and growth of bacteria in motion 45 years ago, when he and Sidney Brenner initiated the study of a mutant with an unusual response of RNA synthesis to amino acid starvation. Without his initial stimulus and the experience gained by trainees in the Stent laboratory, this review would not have been possible.

INTRODUCTION

An important problem of bacterial physiology is how bacteria adapt and optimize their rate of growth in response to different environments. Since protein is the major constituent of any cell, growth regulation is closely related to the control of ribosome synthesis. In fact, the number of ribosomes per amount of total protein present in a growing culture *of Escherichia coli* increases in nearly direct proportion to the growth rate (67, 112). The proportionality would be exact if a constant fraction of ribosomes were elongating proteins at a constant rate (83). Ribosomes are composed of RNA (rRNA) and protein (r-protein). The rate of r-protein synthesis is negatively controlled by free r-proteins, in that excessive accumulation of free r-proteins causes a reduction in the translation and lifetime of r-protein mRNA (38, 39, 63, 77, 81, 84) and thereby reduced synthesis of r-protein. Since the concentration of free r-proteins depends on the concentration of free or nascent rRNA, which titrates r-proteins, this mechanism adjusts the synthesis of r-proteins to the synthesis of rRNA. For this reason, the study of the control of ribosome synthesis centers on the control of rRNA synthesis.

The *E. coli* genome has seven rRNA (*rrn*) operons, each with two tandem promoters, P1 and P2, from which the 16S, 23S, and 5S rRNA transcripts are expressed. The P1 promoters of all seven rRNA operons have the same discriminator sequence, GCGC, bordering identical TATAAT −10 regions (63, 139, 140). Upstream of each of these P1 promoters, there is an activator region with three binding sites for the protein factor Fis (48, 74). Binding of Fis to these sites stimulates expression from P1 (reviewed in reference 63). In addition, rRNA synthesis is inhibited by the nucleotide effector ppGpp (reviewed in reference 23). After correction for position effects in the *E. coli* chromosome, all *rrn* operons are similarly expressed (27; reviewed in reference 63).

Numerous and often conflicting hypotheses about the control of rRNA synthesis in bacteria have been proposed during the last 20 years (see the following section). This control involves a feedback loop that operates in two consecutive steps. First, transcription factors (repressors or activators) and effectors (corepressors, inducers, or molecules binding to RNA polymerase, like ppGpp and nucleoside triphosphate [NTP] substrates) control the activity of *rrn* promoters. Second, the overall activity of these factors and effectors is controlled in response to the balance of the supply of amino acids against the consumption imposed by ribosomal function. In this review, we address the first problem, identification of the factors and effectors that directly interact with either the *rrn* promoter region or the RNA polymerase in a promoter-specific manner. Only when these directly interacting factors and effectors have been clearly identified can one begin to clarify the mechanisms by which the composition of the growth medium affects the activity of these factors. When this second goal is achieved as well, the control of ribosome synthesis can be said to be understood (see the section Perspective and Outlook at the end of this review).

Recently, we addressed the question about the factors controlling the interaction between RNA polymerase and the promoters of the *rrnB* operon with a Michaelis-Menten kinetic approach (143). The underlying rationale for this approach is the fact that any factor that affects these interactions can be defined and measured as a change in the Michaelis-Menten parameters V_{max} and K_m (maximum promoter activity and RNA polymerase concentration at half-maximal activity, respectively). To determine values for these parameters requires a systems biology approach, i.e., the use of mathematical tools to integrate experimental data into a logically consistent conceptional framework.

This review has two parts. First we review the various models for the control of rRNA synthesis in *E. coli* which have been proposed over a period of 45 years. This historical overview is unusually complex because the research that it describes has produced different and often mutually exclusive interpretations of experimental data.

The second part represents an alternative way to describe the same observations from a mathematical rather than historical perspective. This part begins with a description of the theory of transcript initiation, which forms the basis for our kinetic approach to the question of rRNA control. The following sections contain the experimental data used to estimate absolute *rrn* gene activities, an evaluation of the gene activity data in terms of the kinetic properties of *rrn* promoters, and the conclusions from these studies with regard to the control of rRNA synthesis by ppGpp and Fis. At the end, we present a kinetic model for the subreactions involved in *rrn* transcript initiation and the effect of ppGpp in terms of changing rate constants for these different subreactions. It is our hope that this systems biology approach will establish the necessary conceptual framework to resolve the controversies and misunderstandings that have confounded the subject area during past decades.

HISTORICAL OVERVIEW

The control of bacterial ribosome synthesis has been an evolving topic for more than 40 years, beginning in 1958 with the classical work in Maaloe's laboratory that described the changing macromolecular composition of the bacterial cell during growth in different media (67, 112). The various hypotheses that have been proposed over the years about the identity of factors controlling rRNA synthesis as well as about the growth rate-dependent control of the concentration or activity of such factors have recently been described and discussed (143) and are here recapitulated and extended. Because of the importance of the topic, and because of the complexity of the problems involved, the contributions that have been made to this topic from many different laboratories over decades of study are so numerous that it was impossible for us to describe them all. Here we wish to acknowledge our appreciation of the work of a generation of scientists whose contributions to the field, even if not specifically mentioned in our review below, are included in a background of fundamental facts that are often taken for granted.

Primary Control of Ribosomal Protein Synthesis

The first model to explain the control of ribosome synthesis was proposed by Maaloe (83), who made two ad hoc assumptions; one about the factor controlling rRNA synthesis and another about the growth rate-dependent control of this factor: (i) the rate of rRNA transcription is positively controlled by one of the r-proteins and is thereby adjusted to the rate of synthesis of the primarily controlled r-protein and (ii) r-protein promoters are constitutive, and in rich media their activity increases passively due to the repression of amino acid and other biosynthetic operons by exogenous nutrients, which increases the concentration of free RNA polymerase.

Several features implicit in this proposal have been verified: (i) r-protein promoters are constitutive (78); (ii) r-protein promoters are not always saturated with RNA polymerase, so that their activity depends, indeed, on the extent of repression of other genes (78); (iii) specific r-proteins participate in the regulation of ribosome synthesis (63); and (iv) synthesis of rRNA is specifically regulated (see below). However, the constitutivity of r-protein promoters does not imply that r-protein synthesis is unregulated because the mRNAs of r-protein operons contain internal elements that control their elongation, translation, and lifetime (38, 39, 63, 77, 81, 84, 118). The regulatory r-proteins specific to each operon that are not rapidly incorporated into assembling ribosomes bind to these elements, which are often structural mimics of their binding sites on rRNA, and cause transcript attenuation or rapid degradation of the entire mRNA, called retroregulation (77, 84, 118). Since the concentrations of free r-proteins depend on the concentration of free or nascent rRNA, this mechanism adjusts r-protein synthesis to rRNA synthesis, in contrast to the assumption underlying Maaloe's model.

Stringent and Relaxed Responses

The real start and experimental basis for a solution of the problems related to the control of rRNA synthesis was the discovery of the *relA* gene, followed by the elucidation of its function as a ppGpp synthetase. The first important observation was reported over 40 years ago in a study of a bacterial mutant that responds abnormally to amino acid starvation (123). In wild-type bacteria, the accumulation of rRNA immediately ceases if any one amino acid is in short supply. Stent and Brenner (123) concluded that RNA synthesis (actually stable RNA, i.e., rRNA and tRNA synthesis) has a stringent requirement for the presence of all 20 amino acids. Accordingly, the cessation of rRNA synthesis under these conditions became known as the stringent response. In contrast, in the mutant strain, stable RNA accumulation continues for some time during the starvation until it also ceases; i.e., the stringent amino acid requirement was apparently relaxed. This became known as the relaxed response.

When the mutation was mapped (4), the gene was named *relA*. Later, it was found that rRNA synthesis is actually stimulated during the relaxed response but this is obscured because free rRNA becomes unstable in the absence of free r-proteins, so that rRNA accumulation reaches a plateau at a steady state of breakdown and resynthesis (71, 119). An important further step in the elucidation of the amino acid requirement for rRNA synthesis was the finding that not the amino acids themselves are required, but rather the charging of all transfer RNAs with amino acids (90).

Control by Amino Acids

A stimulation of rRNA synthesis, as observed in the relaxed response, can also be induced in wild-type ($relA^+$) bacteria by any inhibition of protein synthesis, e.g., by the antibiotic chloramphenicol (56, 69, 72) or by the inhibition of translation initiation (26). At least a partial explanation for this stimulation was suggested by the finding that the average charging of tRNAs with amino acids actually increases during deprivation for a single amino acid (138); i.e., any inhibition of ribosome function produces a rise in amino acid pools comparable to a nutritional shift-up from a minimal to an amino acid-supplemented medium. This suggested that amino acids play an essential role in the control of rRNA synthesis: the higher the rate of amino acid supply in relation to the capacity of ribosomes to consume amino acids in protein synthesis, the greater the stimulation of rRNA synthesis. However, if all amino acid levels are high except for the one amino acid that is missing, then it is not judicious for the bacteria to make more ribosomes. The mechanism that prevents this involves the function of RelA, which overrides the stimulation by amino acids and inhibits rRNA synthesis under such conditions.

Discovery of ppGpp Synthetase I

The observations described above made it clear that *relA* function is involved in the control of rRNA synthesis. In an attempt to link the reduction in rRNA synthesis during the stringent response to a reduction in the nucleoside triphosphate (NTP) concentrations, Cashel and Gallant discovered instead two new kinds of nucleotides that they named magic spots I and II (MSI and MSII). These nucleotides are formed during amino acid starvation in $relA^+$ but not in *relA* mutant bacteria (21). MSI and MSII were later identified as guanosine

tetra- and pentaphosphate (ppGpp and pppGpp, respectively) (22). Following this discovery, it was soon found that *relA* codes for a ribosome-associated guanosine tetra- and pentaphosphate synthetase (PSI) that converts GDP or GTP in vitro to ppGpp and pppGpp, respectively, when ribosomes are idling at A-site codons in a reaction that depends on the presence of cognate deacylated tRNA (52). In vivo, pppGpp is rapidly converted to ppGpp by a pppGpp-5′-phosphohydrolase (51). These and further observations suggested that ppGpp is involved in the control of rRNA synthesis. Subsequently, it was observed that ppGpp specifically inhibits rRNA synthesis in vitro (50, 65, 66 130–132), presumably by reducing the affinity of the RNA polymerase to stable RNA promoters (50, 53, 65, 66, 98). Thus, unless these in vitro effects are artifactual, they indicate that ppGpp is a direct effector and responsible for at least part of the in vivo inhibition of rRNA synthesis during the stringent response.

Overexpression of *relA* in a strain carrying a *relA* gene linked to the isopropylthiogalactopyranoside (IPTG)-inducible *lac* promoter causes an accumulation of ppGpp accompanied by a rapid decline in rRNA synthesis and growth (126). Mutants with partial resistance to this growth inhibition phenotype were found to have a mutation in the gene for the RNA polymerase β-subunit (126). This suggested that RNA polymerase could be the target for ppGpp action. With biochemical methods, the binding site specific for ppGpp (i.e., for which GDP or GTP do not compete) on the RNA polymerase has now been located by cross-linking at the interface between the β and β′ subunits (112, 129). Recently, the ppGpp-RNA polymerase complex from *Thermus thermophilus* has been studied by X-ray crystallography, where it was found that ppGpp binds near the active center with base-specific contacts between ppGpp and specific cytosine residues in the non template DNA during both transcription initiation and elongation (6).

Based on the term stringent response, reduced promoter activity at elevated levels of ppGpp is now often described as stringent control. However, this term is not clearly defined because, during the stringent response, rRNA synthesis is further inhibited by a greatly reduced RNA polymerase activity (106), presumably due to ppGpp-dependent transcriptional pausing that reduces the concentration of free RNA polymerase (64, 68) and thereby the activity of all unsaturated promoters. Therefore, stringent control may or may not include the effects of specific promoter control by ppGpp.

Differential Inhibition of *rrn* P1 and P2 Promoters by ppGpp

As was mentioned above (see Introduction), all seven rRNA (*rrn*) genes of *E. coli* have two similar promoters, about 120 nucleotides apart, called P1 and P2 (46). It was found that ppGpp preferentially inhibits transcription from P1 (35, 47, 62, 73, 110, 111, 141) in a way that depends on the presence of a discriminator sequence (GCGC) bordering the −10 (TATAAT) recognition sequence of P1 but not P2 promoters (139, 140).

Discovery of ppGpp Synthetase II

The observations described above can explain the inhibition of rRNA synthesis during the stringent response as a result of a *relA*-dependent accumulation of ppGpp. However, the situation during normal exponential growth was unclear, since the growth rate-dependent control of rRNA synthesis is more or less the same in *relA*$^+$ and *relA* bacteria. Since *relA*$^+$ and *relA* bacteria produce similar low basal levels of ppGpp during exponential growth, it was suspected that the *relA1* mutation used in those earlier studies was leaky. However, this idea did not fit the observation that the basal level of ppGpp in *relA* bacteria drops and essentially disappears during the relaxed response. For these and other reasons, it was suggested that *E. coli* might have a second ppGpp synthetase (PSII) that is active during exponential growth (40, 42, 72, 86, 103, 104, 125).

With *lacZ* expression from *rrnB* P1 in a *relA1* strain background as a selectable indicator for PSII-derived basal levels of ppGpp, a search for mutations in the PSII gene was initiated. This search resulted in the isolation of mutants with reduced levels of ppGpp at 30°C and no detectable ppGpp at 43°C. Surprisingly, these mutations mapped in *spoT* (57), a gene that was already known to be coding for the major ppGpp (i.e., magic spot) hydrolase (5, 54, 70, 124), "suggesting that *spoT* encodes both ppGpp degradation and synthesis activities and that these two functions can be independently affected by mutation" (57). This idea was supported by the simultaneous findings in another laboratory that (i) cells with *relA* deletions (i.e., not only the *relA1* mutants) still produce basal levels of ppGpp (137); (ii) cells with *relA spoT* double deletions produce no detectable ppGpp (137); and (iii) *relA* and *spoT* have extensive amino acid sequence similarity (86). Thus, either SpoT is a bifunctional enzyme or the *spoT* polypeptide exists in two different versions that cannot interconvert, i.e., either as a ppGpp synthetase (PSII) or as a ppGpp hydrolase. How the switch between the two activities might be mediated or how the distinct enzymatic activities might be produced is still unknown (see the section Perspective and Outlook at the end of this review).

The basal levels of ppGpp produced during exponential growth in *relA*$^+$ and *relA* bacteria vary with growth rate: the poorer the medium and the slower the growth, the higher the basal (PSII-derived) level of ppGpp (107). By measuring both synthesis and degradation of ppGpp during growth in different media and under different conditions, it could be shown that the PSII activity is highly unstable (40 s average life) and requires continuous protein synthesis (89). Furthermore, the greater the number of different amino acids in the medium, the lower the PSII activity (89). These observations suggest that both PSI and PSII activities are controlled by amino acids, and that both of these enzymes are involved in the control of rRNA synthesis.

RNA Polymerase Partitioning by ppGpp

The ratio r_s/r_t between the synthesis rates of stable tRNA and rRNA (r_s) and of total RNA (r_t) decreases monotonically with increasing levels of ppGpp. At near zero levels of ppGpp, during growth in amino acid-supplemented media or during the relaxed response, r_s/r_t has a value greater than 0.9, i.e., more than 90% of all RNA made in the bacteria is stable rRNA and tRNA and less than 10% is mRNA. In contrast, at increasingly higher levels of ppGpp during growth in minimal media or during the stringent response, r_s/r_t approaches a smallest value of 0.25; i.e., 25% of all RNA synthesized at any instant is stable RNA and 75% is mRNA (8, 17, 107). The

residual rRNA synthesized under the latter conditions originates almost exclusively at the P2 promoters of *rrn* genes (141). It was therefore proposed that ppGpp determines the partitioning of RNA polymerase into stable RNA- and mRNA-synthesizing fractions (17, 107).

The fact that the levels of ppGpp could be experimentally controlled by changing the extent of amino acid starvation in *relA*$^+$ and *relA* bacterial strains (8) shows that ppGpp levels causally affect r_s/r_t, in contrast to a mere correlation between the two. Furthermore, the fact that the ppGpp levels could be continuously varied from near zero (as observed during growth in amino acid-supplemented media) up to the highest levels (as observed during the stringent response) with the same relationship between ppGpp level and r_s/r_t maintained under all conditions supports the idea that ppGpp controls r_s/r_t not only during the stringent response but also during exponential growth in different media (8). These results identified ppGpp as a direct or indirect effector involved in the control of rRNA synthesis.

This conclusion about the control by ppGpp did not address the question of or provide a model for the initial signals involved in this control. This latter issue was addressed by the ribosome feedback models described below, as stated by Cole et al. (26): "Instead of attempting to isolate effectors acting directly on rRNA transcription, our research has concentrated on defining the initial signals leading to regulation of rRNA synthesis."

Ribosome Feedback Models

Before describing the ribosome feedback models, a clarification of the terminology is required. The term feedback regulation (more precisely negative feedback) implies that the value of a controlled parameter is kept nearly constant by a mechanism that senses deviations from the controlled value and generates a signal that leads to an adjustment of this value. This is to be distinguished from a biochemical equilibrium, in which the accumulation of a product inhibits the net rate of the reaction. Since at different growth rates neither the concentrations of total or translating ribosomes nor the rate of ribosome synthesis is constant, it can be asked whether there is any evidence that feedback regulation is involved in the control of ribosome synthesis.

In considering feedback regulation, four basic questions should be addressed. First, which parameter is controlled and held constant? Is it total ribosomes? Or is it only translating ribosomes? Or is it something else? Only once this question is answered can one address the next three questions: What signal is generated when the parameter deviates from its controlled value? How do the deviations produce that signal? And how does that signal adjust the controlled parameter? These questions have generally not been systematically considered in the models described below. For this reason the implied meaning of ribosome feedback has changed several times during the last 20 years, each time with a somewhat different role proposed for ppGpp.

At about the time that the measurements of r_s/r_t and the basal levels of ppGpp during exponential growth at different rates were reported, Nomura and colleagues reported the effect of increased *rrn* gene dosage on *rrn* gene activity by using multicopy plasmids carrying cloned *rrn* operons (60). The increased *rrn* gene dosage was found to reduce the transcriptional activity per *rrn* gene present in the cell. To explain this observation, they suggested that (i) the increased *rrn* gene dosage leads to an excess of nontranslating ribosomes in the cell and (ii) "free, nontranslating ribosomes (i.e., in excess of the amount needed for protein synthesis) inhibit rRNA synthesis." They called this the ribosome feedback regulation model. To distinguish between the possibilities that either (i) some product of *rrn* operons feedback-inhibits rRNA synthesis or (ii) some factor essential for rRNA and tRNA operon transcription (for example, RNA polymerase) is limiting, the authors employed plasmids carrying a deletion in the *rrn* operon leading to the expression of truncated versions of 16S and 23S rRNAs. Using these *rrn* deletion plasmids, they did not observe an inhibition of transcription from the chromosomal *rrn* operons. From this observation, they concluded that the feedback involves products of intact *rrn* operons.

The authors indicated that their efforts to show any possible direct regulatory effects of ribosomes on the transcription from ribosomal promoters in vitro had been negative. Therefore, they considered the possibility that the apparent feedback regulation by free ribosomes is achieved indirectly. To explain the role of RelA, the authors reasoned that "a major effect of ppGpp during amino acid starvation is to inhibit the initiation of protein synthesis" (95). Accordingly, "this inhibition would lead to accumulation of free nontranslating ribosomes in stringent strains which could in turn cause the inhibition of rRNA synthesis." Thus, ppGpp was thought to be an initial effector controlling rRNA synthesis, at least at the high levels of ppGpp accumulating during the stringent response, and free ribosomes would be an additional, either direct or intermediate effector in this control.

With the same *rrn* plasmids as employed by Nomura and coworkers, the effects of *rrn* gene dosage were reinvestigated in greater detail by measuring not only *rrn* transcription in a given medium but also ppGpp accumulation, r_s/r_t, protein synthesis, and plasmid copy numbers during growth in different media (9). Those results indicated that increased *rrn* gene dosage or the presence of plasmids with deletions in their *rrn* operons has complex regulatory effects that involve global changes in growth rate, ppGpp accumulation, mRNA synthesis, and ribosome function that complicate the interpretation of such observations. In contrast to free ribosomes acting as inhibitors, the alternative suggestion was made that increased *rrn* gene dosage would reduce the concentration of free RNA polymerase and thereby reduce the transcription rate per *rrn* gene (19).

According to Cole et al. (26), the observations described above (60) suggested that "the rRNA synthesis rate is modulated through feedback to give the proper rate of ribosome accumulation, as determined by growth conditions." To investigate the next step in this feedback loop, the authors asked whether either free or translating ribosomes influence the RNA synthesis rate. To answer this question, they inhibited the initiation of translation by limiting the cellular concentration of IF2, which results in rapid accumulation of free, nontranslating ribosomes. The expected inhibition of rRNA synthesis was not observed; instead, rRNA synthesis was stimulated. The authors therefore proposed that translating rather than free, nontranslating ribosomes inhibit rRNA synthesis: "In other words, excess ribosomes cause a small increase in translation which in turn generates a signal leading to an eventual decrease in

rRNA synthesis." This became known as the translating ribosome feedback model. Thus, whereas free ribosomes were at first thought to be both the controlled parameter and the controlling signal (i.e., ppGpp was only thought to create free ribosomes during the stringent response [60]), now translating ribosomes were thought to be the controlled parameter, but the question about the nature of the controlling signal was left open; it could have been ppGpp or some other, unknown factor (26).

Ten years later, when initiating nucleoside triphosphates were proposed to be direct effectors controlling rRNA synthesis (44) (see NTP substrate model below), that idea was linked to the translating ribosome feedback model by suggesting that initiating NTPs were the controlling signals: the increased consumption of NTPs during increased translation might reduce the NTP pools, so that rRNA synthesis is reduced (44). However, this cannot explain the increased rRNA synthesis at increased growth rates.

Recently the term feedback has received a new meaning: it was redefined as the specific effects on *rrn* expression associated with changes in *rrn* gene dosage (114). Although the original feedback models addressed the growth rate-dependent control of rRNA synthesis, it was later reported that "the feedback response of *E. coli* rRNA synthesis is not identical to the mechanism of growth rate-dependent control" (135). In that work feedback response and growth rate-dependent control were defined by the changes in LacZ enzyme expression from *rrn* P1 resulting from changes in either *rrn* gene dosage or growth medium, respectively (see the section Current Status of the Field, below). It was then suggested that the gene dosage effects result from associated changes in NTP and ppGpp levels (114).

With regard to this latest use of the term feedback, we note that *rrn* gene dosage is not a parameter that is controlled by or related to feedback. Increased *rrn* gene dosages were only first used, unsucessfully, to generate an excess of free ribosomes. Furthermore, absolute promoter activities were measured in enzyme specific activity units (114), but enzyme expression values obtained from a promoter under different growth conditions do not reflect gene activities (see section below on Gene Expression Observed with Translation or Transcription Assays). The term absolute promoter activity needs to be defined unambiguously as the number of transcripts initiated per unit of time per promoter, not as enzyme specific activity.

At the end of this review, we propose a new feedback model, based on the principles outlined at the beginning of this section. In this new model, the feedback-regulated parameter that is held approximately constant is the function, not the concentration, of ribosomes, and the feedback signal is ppGpp (see Perspective and Outlook, below, for more details).

Passive Control by Free RNA Polymerase Concentration

Whereas the RNA polymerase partitioning model described above assumed that ppGpp was a direct effector in the control of rRNA synthesis, the proponents of the ribosome feedback models excluded such a direct effector role of ppGpp. As a possible way out of this dilemma, Jensen and Pedersen (59) proposed a model of global transcriptional control of stable RNA and mRNA synthesis that followed the ideas of the first Maaloe model described above except that now rRNA promoters, not r-protein promoters, were assumed to be constitutive. They made the following additional assumptions: (i) mRNA promoters have high V_{max}/K_m ratios but low values of V_{max} and K_m; (ii) stable RNA promoters have low V_{max}/K_m ratios but high V_{max} and K_m values; (iii) the Michaelis-Menten parameters of mRNA and stable RNA promoters are unchanged by ppGpp; (iv) elevated levels of ppGpp induce frequent pausing during the transcription of both mRNA and stable RNA genes; (v) ppGpp-dependent transcriptional pausing decreases the free RNA polymerase concentration; and (vi) all ppGpp, including basal levels, originates from PSI as a result of ribosome idling when uncharged tRNA binds to ribosomal A-sites.

Their model implies that mRNA promoters are favored when the concentration of free RNAP in the cell is low and that stable RNA promoters are favored when it is high. When there is excess capacity for protein synthesis in the cell, this will lead to amino acid deprivation and elevated synthesis of ppGpp (by PSI). When the concentration of ppGpp is high, this slows down the rate of transcription of RNA polymerase molecules so that they become sequestered on DNA. This lowers the concentration of free RNA polymerase so that mRNA synthesis is favored in relation to transcription of stable RNA genes. In contrast, when amino acid supply is in excess, the level of ppGpp is low and there is little sequestering of RNA polymerase on DNA. The higher concentration of free RNA polymerase favors transcription of stable RNA genes.

There is support for several but not all of these assumptions (78; see Discussion in reference 18). In particular, their model appears to be valid for the P2 promoters of *rrn* operons (78). However, *rrn* P1 promoters are not constitutive and were later shown to be specifically regulated by ppGpp (56). Furthermore, not all ppGpp is derived from PSI (see above).

The Jensen-Pedersen model was subsequently obscured by the discovery of PSII (see above), by the associated observations on ppGpp-deficient bacteria, and by the NTP substrate model that began to dominate the discussion about the control of rRNA synthesis.

Control of rRNA Synthesis in the Absence of ppGpp

The construction of Δ*relA* Δ*spoT* double deletion (double null) strains devoid of measurable ppGpp was first reported from the Cashel laboratory in 1991 (137). Already a year earlier, the Gourse laboratory had determined the expression of *lacZ* driven by the *rrnB* P1 promoter on a lysogenic λ phage integrated into the chromosome of one of the Cashel double null strains (43). They found that, in the absence of ppGpp, *lacZ* expression increases with growth rate in a manner similar to that in ppGpp-proficient strains. Accordingly, they concluded that "guanosine 3′-diphosphate 5′-diphosphate is not required for growth rate-dependent control of rRNA synthesis in *Escherichia coli*" (43).

With the same double null strains from Cashel but a different *rrnB* P1-*lacZ* fusion, our laboratory later undertook a characterization of RNA and DNA synthesis in *E. coli* strains devoid of ppGpp (58). This consisted of a detailed study of the physiology of ppGpp-deficient strains, including measurements of ribosome concentrations and function, RNA polymerase concentrations and function, chromosome replication data, bulk mRNA gene activities and *rrn* gene activities (in absolute

units), mRNA synthesis rates, r_s/r_t, and *lacZ* expression from *rrnB* P1, all as functions of growth rate. Direct measurements of ribosome synthesis rates were found to increase with growth rate identically in both ppGpp-proficient and ppGpp-deficient strains, which seemed to agree with the conclusion from the earlier study of Gourse's laboratory (based on *lacZ* expression from *rrnB* P1 [43]). However, identical ribosome synthesis rates at a given growth rate between the two strains were expected on theoretical grounds, given that ribosomes in the two strains are equally efficient. This follows from the definition of exponential growth and is independent of any other observations (for an explanation, see equation 3 below under Systems Biology Approach). Therefore, *rrn* gene activity data alone are not sufficient to draw conclusions about the control of rRNA synthesis.

In contrast to the earlier findings (43), our study (58) showed that *lacZ* expression from *rrnB* P1 was approximately constant in ppGpp-deficient strains. The reasons for this discrepancy remain unclear. It was suggested that perhaps differences in the P1-*lacZ* fusion constructs were responsible for it, although a later study with a different P1-*lacZ* fusion ruled this out (141). However, since gene expression data obtained under different growth conditions from a given promoter do not reflect the promoter activities (see Fig. 3 and text below), no conclusion about the control of the promoter was drawn from those *lacZ* expression data. Of more significance was the observation that r_s/r_t values remained approximately constant in the ppGpp-deficient strains (58), whereas they increased with growth rate in ppGpp-proficient strains (107).

Since it had been shown previously that the cytoplasmic level of ppGpp causally determines r_s/r_t (8), the constancy of r_s/r_t in the absence of ppGpp was taken as support for the previous conclusion that ppGpp is involved in the control of rRNA synthesis (58). Furthermore, the original isolation of mutations in the gene for PSII was based on the idea that reduced basal levels of ppGpp should stimulate P1-*lacZ* expression and thereby provide a selectable marker for a mutationally defective PSII gene (57). The fact that this strategy was successful and led to the correct identification of *spoT* as the PSII gene provides strong support for the conclusion that ppGpp is involved in the control of P1. Today, this idea is shared by Gourse and collaborators (see section about New ppGpp Model below).

NTP Substrate Model

Gourse and collaborators measured relative NTP concentrations in bacteria growing at different rates and, in addition, in vitro *rrn* transcription rates at different NTP concentrations. By comparing the in vivo and in vitro observations, they concluded that initiation at *rrn* promoters is controlled by growth rate-dependent changes in the concentrations of the initiating NTPs (44). This became known as the NTP model for the control of rRNA synthesis. As mentioned above, it was proposed that initiating NTPs were the controlling signals in the translational ribosome feedback model and that increased consumption of NTPs during increased translation might reduce the NTP pools, so that initiation at *rrn* promoters is selectively reduced (44).

In contrast to these results, another study showed no apparent growth rate-dependent variations of NTP concentrations in *E. coli* (96). Whereas Gourse's laboratory used alkali for nucleotide extraction after fixation with formaldehyde (44), the other laboratory used formic acid (96). To check whether the different methods might have caused the different results, Schneider et al. (113) compared both formic acid and KOH extraction. They reported that "Although formic acid extraction resulted in higher NTP yields than those obtained by the formaldehyde/alkaline extraction method, relative changes in NTP levels (between strains or between the same strain grown under the different conditions used here) were virtually identical with both extraction methods." Thus, in their hands, NTP levels increased with growth rate independently of the method used. From these and further data, they concluded that NTP sensing by *E. coli* promoters is direct.

However, a repeat of these experiments by Schneider and Gourse (117) gave a contradictory result: "Extraction with formic acid indicated that ATP concentration did not change with growth rate, whereas formaldehyde treatment followed by extraction with alkali indicated that ATP concentration increased proportionally to the growth rate." Sixfold less ATP was found with alkali than with formic acid at a growth rate of 0.8 doubling/h, and threefold less was found during maximal growth in rich medium. Accordingly, the original in vivo NTP concentrations on which the NTP model was based (44) were underestimated in a growth rate-dependent manner. The authors stated: "Because ATP concentrations do not change with growth rate in cells unable to make ppGpp and *rrn* P1 core promoters continue to display growth rate-dependent regulation under these conditions, we conclude that at least one more regulator of *rrn* P1 core promoter activity (in addition to changing concentrations of initiating NTPs and ppGpp) remains to be identified."

The two methods of nucleotide extraction had also been compared previously in connection with the development of a method for quantifying ppGpp in absolute (molar) units (82). In that study, the alkali method was found to be superior to formic acid extraction and apparently 100% efficient. This is to be expected because alkali solubilizes (i.e., saponifies) the lipid membrane and completely lyses the bacteria, so that no extraction is necessary. Apparently, in the Gourse laboratory, the cells were not completely lysed during the alkali treatment, perhaps because insufficient time was allowed for the KOH to work before the sample was neutralized with phosphoric acid. Generally, whenever lysis is incomplete, large cells (which dominate in fast-growing cultures) are preferentially lysed. As a result, the ATP losses were likely to be greatest for slow-growing bacteria, as observed.

To decide finally the question of whether or not intracellular ATP concentrations increase with growth rate, the formic acid and alkali methods used by Schneider and Gourse (117) were complemented with a luciferase assay for determination of the in vivo concentration of ATP under various growth conditions. These measurements of relative ATP concentrations also suggested that the ATP concentration does not vary with the growth rate. However, this assay is associated with a number of caveats. The entry of luciferin into *E. coli* cells was achieved by polymyxin B treatment of the cell populations. Polymyxin B is a bactericidal antibiotic (30) that opens the cell wall for luciferin entry and ATP exit. Since cellular ATP is rapidly turning over and the luminescence assay was performed in the minute time range, it cannot be excluded that the polymyxin B treat-

ment significantly perturbs the rates of synthesis and intracellular consumption of ATP as well as the ATP-ADP ratio. This problem is aggravated by the proposed adjustment of the luminescence peak time to the same value for all bacterial samples by variation of the concentration of added polymyxin B. Moreover, the cytoplasmic ATP concentrations (see following paragraph) are so much higher than the K_m for ATP interaction with their luciferase mutant (0.83 mM) that the assay is expected to be nearly saturated by ATP under the conditions used. In that case, the observed constant luminescence values may not reflect the cellular ATP concentrations.

In summary, it is not yet certain whether the NTP pools are constant or show variations under changing growth conditions. To decide this question, measurements of absolute intracellular concentrations of ATP (in molar units) are needed. This should not be difficult, because the high intracellular ATP concentrations make the UV absorption peak of ATP easily visible (and thus measurable) in chromatographic distributions of cellular nucleotides (82). However, even variable NTP concentrations would not significantly affect the frequency of *rrn* transcript initiation, because they were found to be far above the saturation level for *rrn* transcript initiation (in vitro, 0.8 mM [(44]). This was seen by converting the relative in vivo concentrations obtained by Gaal et al. (44) to absolute concentrations, which ranged from 4 to 10 mM (78). With a different approach, the in vivo concentrations of free NTPs can be estimated from a comparison of the RNA chain elongation rates observed in vitro (V_{max} = 83 nucleotides/s, K_m = 0.63 mM [14, 15]) and in vivo (85 nucleotides/s at all growth rates studied [108, 134]). This comparison suggests a lower limit of 2.5 mM for free NTPs in vivo ($[NTP_f] > 4\ K_m$), still above 80% saturation for initiation.

New ppGpp Model

In recent in vitro studies, Gourse and collaborators showed that (i) the rate of initiation of transcription at the *rrnB* P1 promoter saturates at a much lower concentration of RNA polymerase than the rate of initiation at a promoter for amino acid biosynthetic enzymes; (ii) the rate of open complex formation at *rrnB* P1 but not at the amino acid promoter is reduced by ppGpp; and (iii) the open complex is destabilized at all studied promoters by the action of ppGpp (10, 11). From these data and additional in vivo experiments, they suggested that, in contrast to amino acid promoters, rRNA promoters are always saturated with RNA polymerase. According to this model, an increasing level of ppGpp in the cell reduces the V_{max} for initiation of transcription of rRNA operons, which results in an increased concentration of free RNA polymerase in the cell. This, in turn, enhances the activities of promoters for amino acid biosynthesis and other unsaturated promoters.

In judging the significance of these in vitro observations, we note that the rate of open complex formation is not limiting the P1 promoter activity in vivo and that the free RNA polymerase concentration increases, rather than decreases, with increasing growth rate (see section below on Kinetic Properties of *rrn* Promoters).

The in vitro measurements of Gourse and collaborators (10, 11) were complemented by in vivo measurements of relative P1 promoter activities following nutritional shifts (88). From these experiments, they concluded that "rapid changes in the concentrations of initiating NTPs and ppGpp account for the rapid changes in rRNA expression" after the shift and "changes in initiating NTP concentration dominate regulation during outgrowth from stationary phase, whereas changes in ppGpp concentration are responsible for regulation. . .during exponential phase." This latter statement agrees with the conclusions from earlier studies that established a causal relationship between levels of ppGpp and the rate of rRNA synthesis relative to the total rate of RNA synthesis during exponential growth (8, 17, 107, 108) (see section above on RNA polymerase partitioning by ppGpp).

Kinetic Constants of *rrn* Promoters

Our approach to studying the in vivo control of rRNA synthesis during exponential growth (78, 143) is related to the ideas expressed by Jensen and Pedersen (59) (see above). It is based on a determination of the Michaelis-Menten parameters of RNA polymerase-*rrn* promoter interaction, V_{max} and K_m. If these parameters are constant, the promoter is defined as constitutive, and if they change, the promoter is defined as regulated. The values of V_{max} and K_m include all contributions caused by specific regulatory proteins and effectors as well as general conditions for transcription, such as superhelicity of DNA templates (74, 100, 136) and NTP substrate concentrations (44). Any changes in the values of V_{max} and/or K_m with different growth conditions define promoter-specific control in an unambiguous manner. In this way the previously suggested roles of Fis and ppGpp have been confirmed and quantitated. The results indicate that, during steady-state exponential growth, ppGpp (and its influence on the synthesis and activity of the transcriptional activator Fis) is the only factor involved in the growth medium-dependent control of *rrn* promoter strength (V_{max}/K_m).

The identification of ppGpp as the only effector involved in the control of *rrn* promoter strength does not imply that the growth medium-dependent control of ribosome synthesis is completely understood. The most important questions remaining involve the controls of the ppGpp synthetase activities and of RNA polymerase synthesis in response to changing growth media. However, these questions are usually not addressed in the models about the control of rRNA synthesis, and they are not included in this review.

Current Status of the Field

Despite over four decades of research in numerous laboratories, the problem of the control of ribosome synthesis has remained controversial. Most recently, rRNA promoters were proposed to be subject to three different kinds of control: stringent control as a response to amino acid availability, feedback control as a response to changes in gene dosage, and growth rate-dependent control as a response to changes in the growth medium (88, 115–117). These controls were assumed to involve the effectors ppGpp, initiating NTPs, and others yet to be discovered. The conclusions were based on observed correlations between relative ppGpp or ATP levels, respectively, and relative *rrnB* P1 promoter activities. However, the interpretation of these observations is ambiguous because the various relative values for the concentrations of ppGpp, NTPs,

and promoter activities were measured with different reference units that themselves change during the medium shift conditions studied. Since changing *rrn* gene dosages were not taken into account, relative promoter activities (i.e., reflecting rates of transcript initiation per *rrn* promoter) were actually not measured. Furthermore, to show the in vivo effects of initiating NTPs and ppGpp, it is necessary to measure absolute cytoplasmic concentrations of NTPs and to separate the effects of changing free RNA polymerase concentrations from the effects of ppGpp on RNA polymerase-*rrn* promoter interactions.

The systems biology approach to these problems described in the second part of this review unifies the description of these controls. It is shown that the growth rate-dependent control of the *rrn* P1 promoter is not different from stringent control or the control associated with changing *rrn* gene dosage. The changing P1 promoter strength depends only on the changing cytoplasmic level of ppGpp. In addition, *rrn* gene activities are affected by interdependent changes in RNA polymerase synthesis, free RNA polymerase concentration (depending on the concentrations and activities of all genes in the cell), and chromosome replication-dependent changes in *rrn* gene dosage. It is hoped that the mathematical analysis applied to these problems leads to a better understanding of transcriptional regulation in general and of the control of *rrn* transcription in particular.

SYSTEMS BIOLOGY APPROACH

The main reason for the continuing controversy about the control of bacterial rRNA synthesis is the complexity of the problem that defies traditional molecular biology approaches. In this systems biology approach, we provide a theoretical framework that integrates the experimental data into a consistent picture that should finally help to resolve the controversies and misunderstandings in this field.

For this analysis, it is first necessary to develop the theory and obtain the data to which the theory can be applied. Accordingly, the first three sections below describe the theory of transcript initiation under conditions of balanced, steady-state exponential growth, and the next three sections describe how the absolute activities of the *rrn* P1 and P2 promoters were determined under different growth conditions. Then, in two further sections, the theory is applied to the promoter activity data to find the free RNA polymerase concentrations and kinetic constants of the RNA polymerase-*rrn* promoter interaction. Finally, the meaning of these results with regard to the control of rRNA synthesis is discussed. Based on these results, we present a mathematical model of the process of RNA polymerase binding to *rrn* promoters and the ensuing reactions that lead to transcript initiation, including the effect of ppGpp on these reactions.

Relationship between rRNA Synthesis and Growth Rate

Before describing the theory of transcript initiation, three related background issues are addressed that deal with the relationships between rRNA synthesis and growth rate: (i) the definition of balanced, steady-state exponential growth; (ii) the physiological balance of ribosome concentration and activity that determines the exponential growth rate; and (iii) the so-called square relationship between rRNA synthesis and growth rate.

Definition of balanced steady-state exponential growth. Our work on the growth rate-dependent control of bacterial rRNA synthesis applies to the physiological condition of balanced steady-state exponential growth. Balanced growth means that every component in the medium is present at saturating, nonlimiting concentrations, in contrast to chemostat growth, when one component is growth limiting (83). Steady state means that the bacteria have grown for at least 10 generations in a given medium (i.e., at least a 1,000-fold increase in mass after dilution of an overnight culture). In this condition, the rate of accumulation of every component relative to its total amount in the culture is constant in time. That is, when X is the amount of component X in a culture at time t, then the fractional increase in X per unit time, $(dX/dt)/X$, defines the exponential growth rate:

$$(dX/dt)/X = \ln 2/\tau = (\ln 2/60) \cdot \mu \quad (1)$$

Here τ is the doubling time in minutes, $\ln 2/\tau$ is the exponential growth rate per minute (the reciprocal, $\tau/\ln 2$, is the time required for an e-fold increase), and μ is the growth rate in doublings per hour (equal to 60 min per h/τ). Equation 1 is the basis for several fundamental relationships that define the properties of exponential-phase cultures.

Physiological balance of the controls of rRNA synthesis and ribosome activity. If component X is the total protein P in the cell population, then its amount P (counted as the number of amino acids in peptide chains) can be put into equation 1 instead of X. If, furthermore, the numerator and denominator in the equation are multiplied by the number of ribosomes, N_r, the following relationship between growth rate and ribosome concentration is obtained:

$$\ln 2/\tau = (dP/dt)/P = (N_r/P) \cdot (dP/dt)/N_r \quad (2)$$

This relationship says that the growth rate of an exponential-phase culture ($\ln 2/\tau$) equals the product of the ribosome concentration, given as the number of ribosomes per amount of protein (N_r/P), times the rate of protein synthesis per average ribosome [$(dP/dt)/N_r$]. This expression represents the total rate of protein synthesis (number of peptide bonds made per time unit) divided by the total number of 70S ribosome equivalents in a bacterial culture and has been named ribosome efficiency, e_r (83). The total number of ribosomes includes actively translating ribosomes, free, functional ribosomes, and nonfunctional, immature ribosomes. If the fraction of actively translating ribosomes is defined as β_r and the protein synthesis rate per average active ribosome is defined as the peptide chain elongation rate, c_p, then it follows that $e_r = \beta_r \cdot c_p$, and equation 2 can be rewritten (33) as

$$\ln 2/\tau = (N_r/P) \cdot \beta_r \cdot c_p \quad (2a)$$

This says that bacteria can increase their exponential growth rate by increasing either the concentration of ribosomes (N_r/P), or the proportion of ribosomes actively engaged in translation (β_r; active ribosomes per total number of ribosomes), or the peptide chain elongation rate (c_p; amino acid residues polymerized per minute per active ribosome), or by any combination of changes in these factors. β_r has been found to be approximately constant during exponential growth between 0.6 and 3.0 doublings/h, equal to about 0.8 (41). This means that

80% of all ribosomes are present in polysomes, whereas about 20% represent either free functional or immature nonfunctional 30S and 50S ribosomal particles (80). Therefore, the bacterial growth rate is essentially determined by the two variable factors, ribosome concentration (N_r/P) and peptide chain elongation rate (c_p). Either parameter has limit values, e.g., the bacteria cannot use more than about 25% of their total protein for ribosomal protein, and individual ribosomes cannot synthesize protein faster than at about 21 amino acids per second at saturation with their substrates (16). Below such limits, however, bacteria must balance their metabolic activities between the production of either ribosomes or factors and substrates involved in ribosome function.

It has been argued on theoretical grounds that the observed balance (see Fig. 5a and b below) serves to maximize the growth rate in different media (37). It appears that the whole bacterial metabolism is geared to supply activated amino acids (aminoacyl-tRNAs) at a rate sufficient for the ribosomes to function at nearly maximal c_p. If the conditions are such that this is not possible, then c_p drops below its maximal value. This stimulates the activities of the ppGpp synthetases (see Historical Overview, above), which produce the signal molecule ppGpp (22, 57, 137), which specifically reduces transcription of *rrn* operons (23, 143). The ensuing reduced ribosome synthesis leads to a new balance at which fewer ribosomes function at only a slightly reduced but still nearly maximal rate. In this manner, ribosome function is monitored to achieve the particular balance between ribosome synthesis and function that maximizes the fitness of bacterial populations.

The following review of our analysis of the growth rate-dependent control of rRNA synthesis describes only the in vivo effects of ppGpp and ppGpp-dependent Fis synthesis on the initiation of transcription from *rrn* promoters and excludes a discussion of the control of ppGpp levels by the growth medium. The latter involves complex controls of cytoplasmic amino acid levels, of ribosome function, and of the synthesis and turnover rates of ppGpp. These phenomena are not yet fully understood and must await further studies before they can be brought into a full picture of the control of ribosome synthesis by the growth medium (see the section about Perspective and Outlook at the end of this review).

Square relationship between rRNA synthesis and growth rate. To define the control of rRNA synthesis, it is frequently stated that rate of rRNA synthesis increases with the square of the growth rate (60, 111), or most recently, "rRNA synthesis is proportional to the square of the culture's growth rate. The molecular basis for this phenomenon, called growth rate-dependent control, still remains unresolved, however" (117). In all these cases, the reference unit needed to define the rate of rRNA synthesis was not given (e.g., rate per gene, per cell, per mass unit, or per culture volume). However, the authors consistently cite Maaloe's work for their statement. Since both cell size and DNA content increase dramatically with growth rate (112), Maaloe suggested using the reference unit per genome equivalent of DNA (also referred to as per genome for short) rather than per cell to measure macromolecular components (83).

During moderate to fast growth, the amount of RNA per genome in *Salmonella* spp. and in *E. coli* B/r was found to increase in direct proportion to the growth rate (33, 112). This proportionality implies that the rate of RNA accumulation per genome $[(dr/dt)/G]$ increases with the square of the growth rate, i.e., with μ^2 (see equation 1 above). However, this reflects the control of both RNA and DNA synthesis, i.e., the initiation and velocity of chromosome replication (13, 16, 55). Therefore, the relationship is altered in bacterial mutants that exhibit aberrant control of DNA replication but have normal control of rRNA synthesis (25), so that this square relationship is unsuited to define the growth rate-dependent control of rRNA synthesis.

The relationship has been restated with the reference per amount of protein, e.g., "the synthesis of rRNA per unit amount of protein increases with the square of the growth rate and this phenomenon is called growth rate-dependent control of rRNA synthesis" (135). This statement derives from equation 2 above as follows. Each ribosome contains the equivalent of one *rrn* transcript, so that N_r/P equals the number of rRNA transcripts per amount of protein, r/P. When r is used instead of X in equation 1 and instead of N_r in equation 2a, these two expressions together with the definition $e_r = (dP/dt)/N_r$ can be used to write the rRNA synthesis rate per amount of protein as

$$(dr/dt)/P = (1/e_r) \cdot (\ln 2/t)^2 = (1/e_r) \cdot (\ln 2/60)^2 \cdot \mu^2 \qquad (3)$$

Thus, if e_r were constant and independent of the growth rate, then the rate of rRNA synthesis per amount of protein, $(dr/dt)/P$, would indeed be proportional to μ^2. However, e_r has been determined from measurements of RNA and protein in absolute units; all such measurements have indicated that e_r is not constant but increases with increasing growth rate and approaches a maximum value (20, 33, 142) (see Fig. 5 below). Therefore, the square relationship does not hold. Moreover, the relationship (3) is based entirely on equation 1, which is only a logical consequence of exponential growth and therefore cannot reflect the workings of a control mechanism. Rather, the analysis of this control needs to be developed from the theory of transcript initiation applied to in vivo conditions (see below).

Theory of Transcript Initiation under In Vivo Conditions

The theory of transcript initiation has been derived in the past from in vitro transcription studies with purified RNA polymerase and promoter-carrying DNA fragments (see the review by Record et al. [101]). In the following, an extended version of this theory that applies to the in vivo situation is presented (78). During exponential growth in vivo, the transcription of a given gene is initiated and terminated at a constant rate, and the concentration of free RNA polymerase is maintained at a steady-state level in a manner that has not been possible to duplicate in vitro.

Reactions involved in transcript initiation. The reactions involved in the initiation of transcripts at a given promoter can be described by the following scheme (78):

$$\mathrm{R} + \mathrm{P} \underset{k_{-\mathrm{I}}}{\overset{k_{\mathrm{I}}}{\leftrightarrow}} \mathrm{RP}_{c1} \underset{k_{-\mathrm{II}}}{\overset{k_{\mathrm{II}}}{\leftrightarrow}} \mathrm{RP}_{o2} \underset{k_{-\mathrm{III}}}{\overset{k_{\mathrm{III}}}{\leftrightarrow}}$$

(reversible)

$$\mathrm{RP}_{\mathrm{init}(1,m)} \xrightarrow{k_{\mathrm{IV}}} \mathrm{TC}_{(m+1,n)} \xrightarrow{+\sigma,\ k_{\mathrm{V}}} \mathrm{TC}_{(n+1)} + \mathrm{P}$$

(irreversible)

In this scheme, R is the free RNA polymerase, P is the free promoter, RPc_1 is the closed complex, RPo_2 is the open complex, $RP_{init(1,\ m)}$ is the initiation complex, including abortive initiations, when the transcript has a length of less than m nucleotides ($m = 10$), $TC_{(m+1,\ n)}$ is the transcription complex after the release of σ (i.e., completion of the transition between initiation and elongation) at m nucleotides when the transcript has a length of between $m + 1$ and n nucleotides ($n = 50$), and $TC_{(n+1)}$ is the transcription complex after promoter regeneration when the polymerase has moved $n + 1$ nucleotides away from the promoter. The first four reactions are described by Record et al. (101). They were originally derived for the in vitro transcription of promoter fragments, where the polymerase falls off at the end of the template immediately after the release of the σ factor. Reaction 5 has been added by Liang et al. (78) to describe the in vivo situation, where the RNA polymerase has to move at least 50 nucleotides away from the promoter to make sufficient room for binding of the next polymerase to the promoter. This last kinetic step limits the maximal activity of rRNA promoters and other promoters with very short promoter clearance times.

The rate constants associated with these reactions can be understood from their reciprocals. Thus, $1/(k_I[R_f])$ is the average time required for an RNA polymerase with free concentration $[R_f]$ to bind the promoter, $1/k_{II}$ is the average time for the RNA polymerase to go once from RP_{c1} to RP_{o2}, $1/k_{III}$ is the average time for it to go once from RP_{o2} to $RP_{init(1)}$, $1/k_{IV}$ is the time required for it to go from $RP_{init(1)}$ to $TC_{(m+1)}$, $1/k_V$ is the time required to sufficiently elongate the transcript to regenerate a free promoter, $1/k_{-I}$ is the average time the polymerase remains in the closed complex before dissociating again from the promoter, $1/k_{-II}$ is the average time the open complex exists before reverting to the closed complex, and $1/k_{-III}$ is the average time the initiation complex exists before reverting to the open complex.

These eight rate constants (i.e., five forward and three backward reactions) determine the activity of a promoter under a given condition. The values for some of these rate constants have been estimated in vitro but are often incompatible with the situation in vivo. For example, in vitro, the time required for the formation of the open complex at the *rrnB* P1 promoter at saturation with RNA polymerase has recently been found to be 25 s (10). In vivo, this reaction needs to be at least 100 times faster in order to account for the rate of initiation at *rrn* promoters in rapidly growing cells (143) (see Mathematical Modeling *rrn* Transcript Initiation at the end of this review). For these reasons, we have argued that, in vivo, the reactions leading to promoter clearance and promoter regeneration rather than those leading to open complex and initiation complex formation (see the scheme above) become limiting for *rrn* promoter activity. In the following, we define the RNA polymerase-promoter interactions in terms of Michaelis-Menten parameters and use the scheme above as a support for interpretations and, in some cases, to constrain the parameter values.

Promoter activity under steady-state conditions. Under steady-state in vivo conditions, the activity, V, of a given promoter depends on the promoter-specific Michaelis-Menten parameters V_{max} and K_m and the concentration of free RNA polymerase, $[R_f]$:

$$V = V_{max}\{1/(1 + K_m/[R_f])\} \quad (4)$$

V is the rate of transcript initiation at the promoter (initiations/minute), V_{max} is the maximum activity at promoter saturation (initiations/minute), $[R_f]$ is the concentration of free RNA polymerase, and K_m is the concentration of free RNAP at half-maximal rate. The factor on the right side, $1/(1 + K_m/[R_f])$, represents the probability that the promoter is occupied by an RNA polymerase. For $[R_f] \rightarrow \infty$, this factor approaches 1.0, so that V approaches V_{max}. The values for V_{max} and K_m include the effects of all rate constants involved in transcript initiation (see below). In the following, it is explained how the values for V, V_{max}, K_m, and $[R_f]$ can be estimated under in vivo conditions.

Effects of varying free RNA polymerase concentrations. The effects of a changing free RNA polymerase concentration on the rate of transcript initiation at a given promoter are seen best by writing equation 4 in its reciprocal form:

$$1/V = 1/V_{max} + (K_m/[R_f])/V_{max} \quad (5)$$

Defining t_i as the average time between two transcript initiations and equal to $1/V$, t_{min} as the average minimum time between two transcript initiations and equal to $1/V_{max}$, and t_b as the average time required for RNA polymerase binding per transcript and equal to $(K_m/V_{max}) \cdot 1/[R_f]$ gives

$$t_i = t_{min} + t_b \quad (6)$$

Relationship 6 implies that the average time between two transcript initiations equals the sum of the average minimum time, t_{min}, between initiations observed under conditions of promoter saturation with polymerase plus the average time t_b required for RNA polymerase binding. The time t_b is proportional to the reciprocal of the free RNA polymerase concentration $[R_f]$ and is zero when the RNA polymerase concentration saturates the promoter.

This is illustrated in Fig. 1 for two constitutive *E. coli* promoters, ribosomal protein promoter P_{spc} and the P2 promoter of *rrnB* (78); how the values used in this figure were obtained will be explained in the sections below. P_{spc} is one of the strongest mRNA promoters (78) and t_{min} for P_{spc} is seen to be about 2 s. In contrast, t_{min} for the rRNA P2 promoter is four times less, i.e., about one initiation every 0.5 s. However, at a given nonsaturating concentration of free RNA polymerase, the binding times for P_{spc} are shorter (3 s during growth in glycerol minimal medium; last point on the curve) than for *rrn* P2 (7 s). This means that at low concentrations of free RNA polymerase, i.e., during slow growth in poor media, P_{spc} activity is greater than *rrn* P2 activity, whereas at high RNA polymerase concentrations during fast growth in rich media, P2 activity is greater. This implies that the activity of a promoter under a given condition does not always measure its strength (see the definition of the term promoter strength below in the section Control of Promoter Strength), as assumed by McClure (85). In general, rRNA promoters appear to be binding limited with fast promoter clearance times, so that they are only saturated at high concentrations of free polymerase (34). In contrast, mRNA promoters have long promoter clearance times and become saturated at lower concentrations of polymerase (78).

Effects of varying promoter concentrations. The cytoplasmic concentrations of all bacterial promoters vary during the cell cycle as a result of DNA replication and during growth in

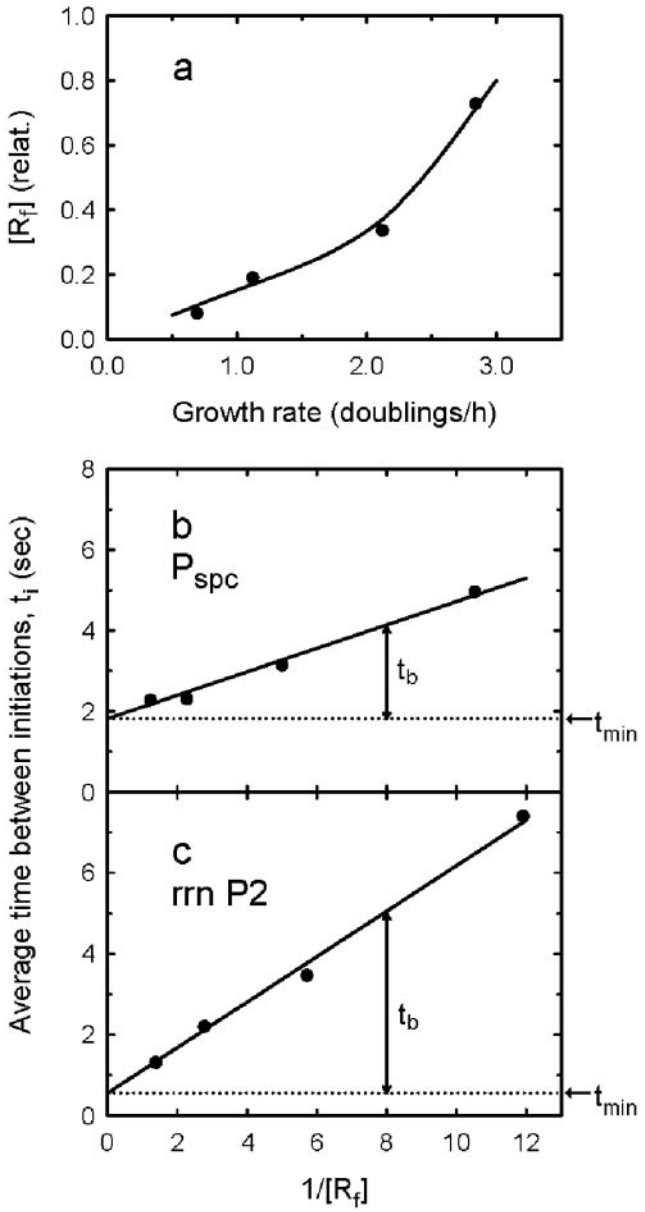

FIG. 1. Relative cytoplasmic concentration of free RNA polymerase $[R_f]$ as a function of growth rate (a) and average time between two transcript initiations t_i at the promoters P_{spc} (b) and $P2_{rrnB}$ (c) as a function of $1/[R_f]$ (data from reference 78). Dotted line, t_{min} at $[R_f] = \infty$; arrowheads, average RNA polymerase binding times t_b at $1/[R_f] = 8$ (relative value, shown as an example).

different media as a result of the growth medium-dependent control of DNA replication (13, 28, 36, 55). The theoretical relationship between promoter concentration and $[R_f]$ depends on the kinetic properties of all promoters present in the cell and on the lengths of transcripts and rate of transcription of the associated genes (19). When this theory is applied to an idealized *E. coli* cell with a given concentration of total RNA polymerase (2,000 molecules per cell) and a variable number of mRNA genes (0 to 400 per cell, with a length of 1,500 nucleotides and typical V_{max} and K_m values), the following relationships become apparent (Fig. 2). (i) At low DNA concentrations (below 50 genes per cell), the increasing concentration of genes and promoters causes an increasing rate of total transcription (Fig. 2c). The rate of total transcription is then limited by the total DNA available, and all promoters are nearly saturated with polymerase and active at near their maximum rate (Fig. 2b). In addition, most of the total polymerase is in the form of free RNA polymerase (Fig. 2a). This condition may be described as excess RNA polymerase. (ii) When the DNA and promoter concentrations are gradually increased to above 200 genes per cell, the total rate of transcription reaches a plateau (Fig. 2c), but the free RNA polymerase concentration (Fig. 2a) and the activity per promoter (Fig. 2b) continue to decrease. Such conditions, when the total rate of transcription is limited by the total RNA polymerase, may be described as excess DNA. Under these conditions, free RNA polymerase is only a small fraction of the total, and the major fraction of RNA polymerase is either bound to a promoter or involved in transcript elongation. Excess DNA appears to be the typical in vivo condition in bacterial cells (19, 122, 143).

Consistent with the model shown in Fig. 2, numerous observations have shown that the rate of transcription per *rrn* gene correlates negatively to the concentration of *rrn* genes. That is, the rate per *rrn* gene decreases when the *rrn* gene concentration increases by replication during the cell cycle (32), by a mutation that alters the control of replication initiation (25), or by adding *rrn* operons cloned on plasmids (9, 60). Conversely, the rate increases when some of the seven *rrn* operons are deleted (27). The latter results, involving *rrn* plasmids or *rrn* deletions, were interpreted as feedback control of rRNA synthesis (115) (see Historical Overview above). Based on the theoretical arguments given above, we have suggested that they are in fact caused by gene dosage-dependent changes in $[R_f]$, such as illustrated in Fig. 2 (9, 19).

Rate constants for the reactions involved in transcript initiation. The two time parameters on the right side of equation 6, t_{min} and t_b, are related to the eight rate constants of the reactions involved in transcript initiation (above) as follows (78) (also see Mathematical Modeling of *rrn* Transcript Initiation below):

$$t_{min} = 1/k_{II}[1 + k_{-II}/k_{III}(1 + k_{-III}/k_{IV})] + 1/k_{III}[1 + k_{-III}/k_{IV}] + 1/k_{IV} + 1/k_V \quad (7)$$

$$t_b = n_b/([R_f] \cdot k_I) = (n_b/k_I) \cdot 1/[R_f] \quad (8)$$

$$n_b = K_m/(V_{max} \cdot k_I) \quad (9)$$

Here n_b is the average number of binding events per successful initiation. In relationship 7, the factor expressions in square brackets in the first two terms on the right side represent the average number of times that the open complex and initiation complex, respectively, have to be formed for one successful transcript initiation (78) (see also equations A8 and A9 in the section Mathematical Modeling of *rrn* Transcript Initiation below). The reciprocals of these numbers represent the probabilities that the formation of an open complex or initiation complex, respectively, leads to a successful initiation. If, for example, a reaction leads to transcript initiation with 50% probability, then it has to be repeated on average twice per initiation event. If the backward rates k_{-II} and k_{-III} are small in relation to the respective forward rates k_{II} and k_{III}, these

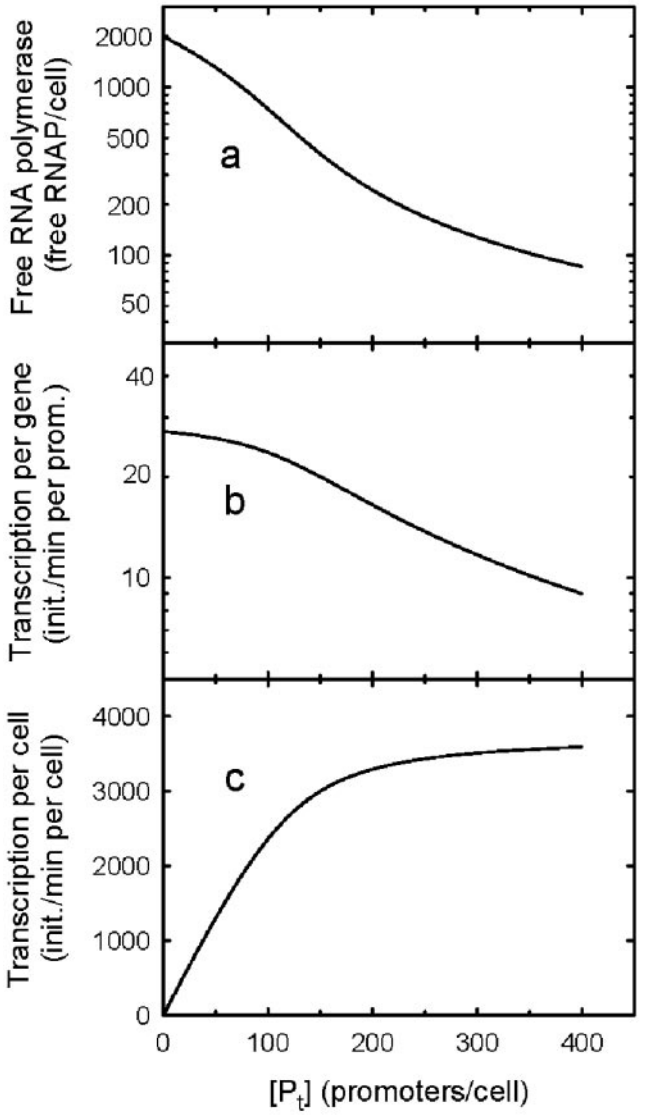

FIG. 2. Effect of varying gene concentration on the total rate of RNA synthesis, rate of transcription per gene, and concentration of free RNA polymerase (data from reference 19). The relationships are derived from an idealized cell, in which all promoters are identical and the transcription times of all genes are equal. The volume of the cell is 1 unit, and concentrations are given as numbers of molecules per cell. The cell contains 2,000 RNA polymerase molecules, and the number of promoters per cell $[P_t]$ is varied between 0 and 400 (abscissa in all panels). V_{max} = 30 initiations/minute per promoter, and K_m = 200 molecules/cell. All transcripts are 1,500 nucleotides long, and the RNA chain elongation rate is 50 nucleotides/s. (c) The ordinate is the total steady-state rate of transcription, $i = V \cdot [P_t]$, measured as transcripts per minute per cell. (b) The ordinate is the steady-state rate, V, of transcription for one promoter measured as transcripts per minute per promoter and calculated from equation 4. (a) The ordinate is the free RNA polymerase concentration, $[R_f]$, calculated from equation 7 in reference 19. For panels b and c, the ordinates are shown in log scale to illustrate how V and $[R_f]$ approach zero as $[P_t]$ increases and the total rate of transcription per cell, i, approaches its plateau value.

probabilities approach the maximum of 1.0, and the number of times the reaction occurs per initiation approaches the minimum of 1.0 (see section Mathematical Modeling of *rrn* Transcript Initiation below for further details and discussion).

The values of k_{IV} and k_V depend on the RNA chain elongation rate and on the number of nucleotides, n, that an initiating RNA polymerase has to move away from the promoter to allow the next RNA polymerase to bind. The nascent rRNA chains are known to be elongated at a rate of about 5,400 nucleotides/min (134), and a realistic value for n is 50 nucleotides (142). Accordingly, we find for the reciprocal of the sum $1/k_{IV} + 1/k_V$ [i.e. $k_{IV}k_V/(k_{IV} + k_V)$] a value of about 110 initiations/min (5,400/50). Since the observed rRNA promoter activities approach this value during growth in rich media (see Fig. 7 below), we have argued that, for rRNA promoters, the values for k_{II} and k_{III} are small compared to $k_{IV} + k_V$, so that for rRNA promoters, V_{max} corresponds to about 110 initiations/min (142).

A fast formation of the open complex and of the initiation complex for rRNA transcript initiation generally implies that the backward reactions from the open complex or the initiation complex are infrequent. However, if k_{II} and k_{III} have very high values, then even several repetitions of these reactions due to their reversibility (high values for k_{-II} and/or k_{-III}) would not significantly add to the total time required for a successful initiation, since initiation is limited by the chain elongation steps represented by k_{IV} and k_V. But, if the forward reactions are slowed down (decreased k_{II} and/or k_{III}) as a result of regulation (e.g., by the action of ppGpp at the P1 promoter), then higher values for k_{-II} and/or k_{-III} would amplify this regulatory effect because of the ensuing repetitions of the slowed forward reactions. This could be a mechanism whereby ppGpp reduces *rrn* P1 promoter strength (see Mathematical Modeling of *rrn* Transcript Initiation below).

Transcriptional Control of Gene Expression

Constitutive and regulated promoters. If the V_{max} and K_m of a promoter remain constant under various conditions of growth, we define the promoter as constitutive. If, in contrast, V_{max} and/or K_m varies, we define it as regulated. With these definitions, variations in the activity of constitutive promoters reflect only variations in $[R_f]$, whereas variations in the activity of regulated promoters reflect variations in $[R_f]$ and/or in their Michaelis-Menten parameters. As will be shown below, the *rrn* P1 promoter is regulated and the *rrn* P2 promoter is constitutive.

Control of promoter strength. At very low, nonsaturating concentrations of free RNA polymerase ($[R_f] \rightarrow 0$), a given promoter shows a certain rate of transcript initiation, V. The greater this rate at a given $[R_f]$, the stronger the promoter. For small values of $[R_f]$ ($[R_f] \ll K_m$), the Michaelis-Menten equation 4 above becomes

$$V([R_f] \rightarrow 0) = (V_{max}/K_m) \cdot [R_f] \quad (10)$$

Under these conditions, promoter activity becomes directly proportional to $[R_f]$. The factor of proportionality, V_{max}/K_m, defines the promoter strength. Growth rate-dependent changes in V_{max}/K_m for a given promoter are a measure of its control.

This definition of promoter strength differs from the definition given by McClure (85): "The term promoter strength refers to the relative rate of synthesis of full-length RNA product from a given promoter, and initiation frequency expresses the same idea in absolute units of reciprocal time (e.g., 10 chains/min, once/generation, etc.)." According to that defini-

tion, promoter strength equals V, or V_{max} if the promoters are saturated with polymerase, rather than V_{max}/K_m.

Control by exogenous and endogenous effectors. Promoters may be controlled by exogenous and endogenous effectors. For example, the *lac* operon and amino acid biosynthetic operons are controlled by exogenous lactose or amino acids, respectively, in the growth medium. Lactose acts as an inducer and the amino acids as corepressors. The control can in these and similar cases be studied by varying the concentration of the effector in the growth medium, often with very small effects on the growth rate or on the general physiology of the cells. This is the case when induction or repression involves only one or a few operons, e.g., when lactose or a single amino acid is added to a culture growing in glucose minimal medium or, in the classical example, when the *lac* operon is controlled by the concentration of the gratuitous inducer isopropylthiogalactopyranoside (IPTG) in the growth medium. Under these conditions the concentration of free RNA polymerase in the bacterial cytoplasm can be assumed to remain essentially constant, i.e., unaffected by the effector concentration in the medium, so that the observed changes in gene expression reflect a specific control of the promoter. Here the changing gene expression at increasing concentration of the exogenous effector shows a control of V_{max} that depends on the probability that an effector is bound to the repressor.

Genes controlled by endogenously generated signals include the operons for rRNA, r-proteins, RNA polymerase subunits, ribosomal factors, and others. In general, these endogenous signals can only be varied by changing the composition of the growth medium, which also changes the growth rate. In these cases, any observed changes in gene activity may reflect either a specific control of the promoter or changes in free RNA polymerase concentration associated with changes in the growth rate. In addition, the observed gene expression is affected by the control of the reference unit used, total protein or total RNA. For example, growth rate-dependent control might be observed as a change in the amount of enzyme per total protein (enzyme specific activity) or in the amount of specific mRNA per total RNA with quite different results (see the following section). These complications make it difficult to interpret expression data from endogenously controlled genes.

Gene expressions observed with translation or transcription assays. An example of the ambiguities associated with the study of endogenously controlled promoters is illustrated in Fig. 3, which shows the growth rate-dependent control of β-galactosidase expressed from the constitutive ribosomal protein promoter P_{spc} (78). With a translational or enzyme activity assay, *lacZ* expression from P_{spc} is seen to decrease with increasing growth rate (Fig. 3a). In contrast, with a transcriptional assay based on hybridization with a probe specific for *lacZ* mRNA, the proportion of *lacZ* mRNA per total mRNA is seen to increase with increasing growth rate (Fig. 3b). This discrepancy is not apparent when only one or the other kind of assay is used. How, then, can the discrepancy be explained? One possibility seems to be that there is translational control of *lacZ* mRNA. However, this is ruled out by experiments showing that the rate of translation of *lacZ* mRNA is almost constant and independent of the growth rate (Fig. 3c).

The explanation for the discrepancy is found from a fourth type of measurement; the rate of translation per average bulk mRNA increases steeply with increasing growth rate (Fig. 3d). This shows that the constitutive mRNAs made during fast growth in rich media have more efficient ribosome-binding sites than the average repressible mRNAs made during slow growth in poor media. As a result, the bulk mRNAs made during fast growth compete more efficiently for ribosome binding and translation, so that, despite the increasing abundance of *lacZ* mRNA (Fig. 3b), the translation products of *lacZ* mRNA become a decreasing proportion of total protein (Fig. 3a).

Such difficulties in the interpretation of gene expression data obtained at different growth rates, as illustrated in Fig. 3, are a primary reason for the continuing uncertainty about the growth rate-dependent control of rRNA synthesis in *E. coli*. This dilemma can only be resolved by determination of the absolute activities of rRNA promoters (initiations per minute per promoter) as functions of growth rate. In the following, it is described how this can be done.

Transcriptional Activity of *rrn* Operons

In the preceding section, the theory of transcript initiation and of promoter control under in vivo conditions of steady-state exponential growth has been reviewed. Before this theory can be applied to the growth rate-dependent control of rRNA synthesis, the absolute activities of the two *rrn* promoters have to be determined as transcripts initiated per minute per promoter. In this section we describe the methods used for this purpose and the results obtained.

Rationale for the method. The in vivo rate of transcription from the promoter of any chosen gene X can be found in absolute units (transcripts per minute per promoter) from two kinds of measurement. First, it is necessary to find the absolute activity of a suitable reference gene or operon with a stable transcript that can be accurately quantified. For this purpose we have chosen the *rrn* operons, since rRNA is stable under most conditions of exponential growth. Then, relative expressions from the promoters of gene X and from the reference (*rrn*) operon have to be determined. To this end, we have used P_x-*lacZ* and P_{rrn}-*lacZ* fusions with either transcription or translation assays for *lacZ* (78, 141). The absolute activity of gene X, V_x, in transcripts per minute per promoter, is then found as the product of the absolute activity of the *rrn* reference operon, V_{rrn}, and the quotient of the relative expression values, $E_{x\text{-}lac}/E_{rrn\text{-}lac}$ as follows:

$$V_x = V_{rrn} \cdot E_{x\text{-}lac}/E_{rrn\text{-}lac} \quad (11)$$

The problems highlighted in Fig. 3, i.e., the particular units used to measure gene expression (e.g., LacZ enzyme activity per total protein or *lacZ* transcripts per total RNA), cancel in the quotient of expression values. In the following three sections we describe (in three steps) how this method is applied to the P1 and P2 promoters of the *rrnB* operon.

Measurement of protein and nucleic acids. The transcriptional activity of an average *rrn* operon (V_{rrn} in equation 11 above, measured in transcripts per minute per promoter) was obtained from four kinds of primary measurements, each in absolute units per mass unit of culture (Fig. 4): (i) total protein (amino acid residues per unit of optical density at 600 nm [OD_{600}]); (ii) total RNA (RNA nucleotides per OD_{600}); (iii) total DNA (genome equivalents per OD_{600}; 1 genome equiv-

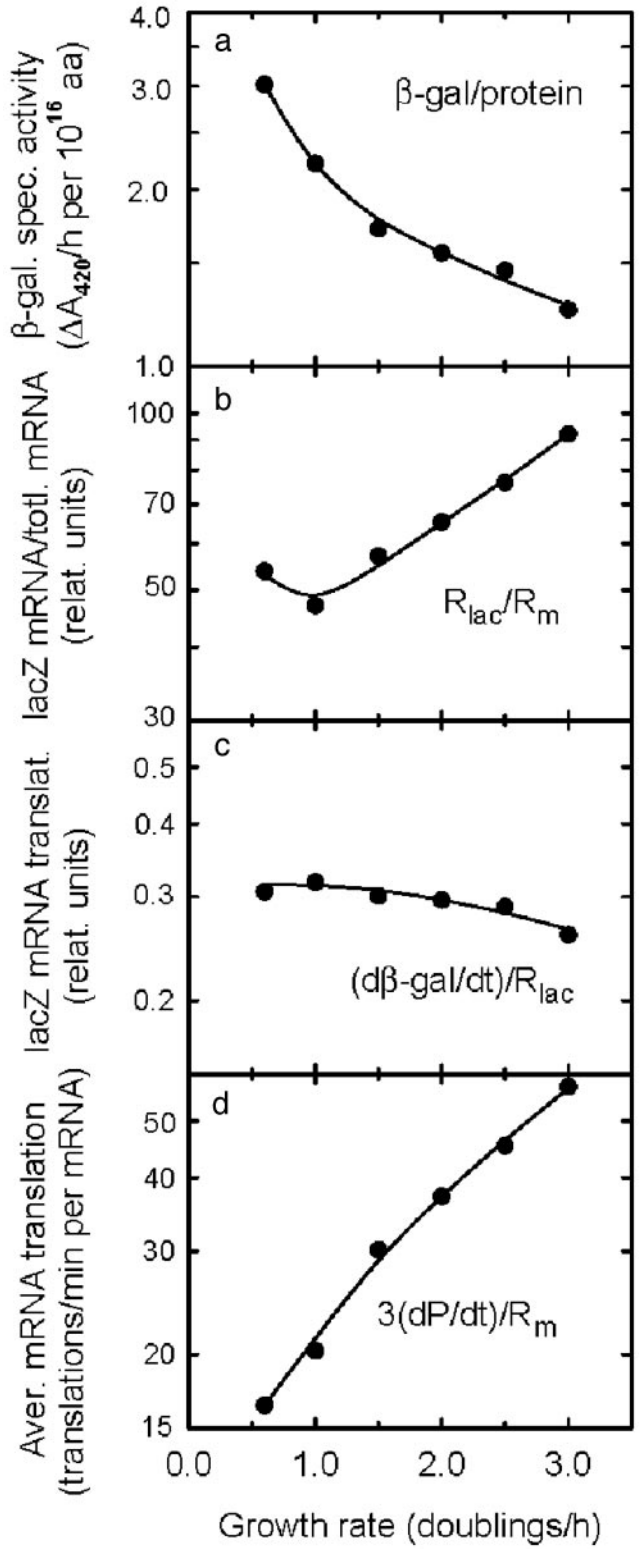

FIG. 3. Growth rate dependence of the β-galactosidase specific activity expressed from P_{spc} in *E. coli* and of transcription and translation parameters that determine this specific activity (data from reference 76); see the text for further explanations. (a) β-Galactosidase specific activity. (b) Relative abundance of *lacZ* mRNA in total mRNA. (c) Rate of translation initiation of *lacZ* mRNA in relative units. (d) Average rate of translation initiation of total (bulk) mRNA in translations per minute per average mRNA molecule. This rate can be found either from the total rate of protein synthesis per amount of mRNA [$(dP/dt)/R_m$] or from the peptide chain elongation rate and the average distance of ribosomes on the mRNA (see Table 3 in reference 16).

alent = 4.2 Mbp); (iv) number of copies of the replication origin (*oriC* copies per OD_{600}). The determination of protein is not directly required for finding the *rrn* gene activity, but it is useful in interpreting observations when the growth rate is changed (e.g., as a result of Fis deficiency) or when enzyme activity data are evaluated. Since the methods used for these determinations, essential for analysis of gene expression in general and growth medium control in particular, have not been generally employed in other laboratories, they are summarized here for convenience.

Total RNA in absolute units was obtained from the A_{260} of RNA hydrolysates, the base composition of average *E. coli* RNA, and the molar extinction coefficients of the RNA bases. Absolute values for protein and DNA were obtained as averages over data obtained with colorimetric assays corrected for nonlinearity and performed at several time points during exponential growth of the cultures. The number of copies of *oriC* was obtained from the amount of DNA that accumulates after stopping the initiation of replication with antibiotics and allowing the ongoing rounds of replication to be completed, after measuring, and taking into account, a short delay between the cessation of protein synthesis and cessation of initiation. The exponential growth rate was determined from the increase in the OD_{600} of the culture (also corrected for nonlinearity). These methods are described in detail by Bipatnath et al. (13).

The results of such measurements, plotted as functions of growth rate, are illustrated in Fig. 4 (from reference 142) for two isogenic strains; one wild type and the other carrying a deletion in the gene for the factor, Fis, that stimulates transcription from the P1 promoters of *rrn* operons. Each strain was grown in four different media (glycerol minimal, glucose minimal, glucose amino acids, and Luria-Bertani [LB] medium) to obtain a range of growth rates between approximately 0.7 and 3.0 doublings/h. The absence of Fis is seen to have little effect on the accumulation of protein and RNA at a given growth rate but leads to significantly lower DNA and *oriC* concentrations at all growth rates, reflecting a stimulating effect of Fis on the initiation of DNA replication at *oriC* that affects the *rrn* gene dosage (see below).

Calculation of *rrn* transcriptional activities. From the primary data of total protein, RNA, DNA, and replication origins (as in Fig. 4), four further parameters were calculated (Fig. 5): (i) the quotient RNA per protein (RNA nucleotides in total RNA per amino acid residue in total protein); (ii) the peptide chain elongation rate (amino acids polymerized per second per active ribosome); (iii) protein per *oriC* (amino acids/*oriC*); and (iv), the (wanted) *rrn* gene activity in absolute units (initiations per minute per *rrn* operon). The first two parameters are related to ribosome concentration and function (see equation 2a above). Protein per origin, also called initiation mass, is a measure for the control of DNA replication (13, 36). The *rrn* transcription activity was obtained by four sequential conver-

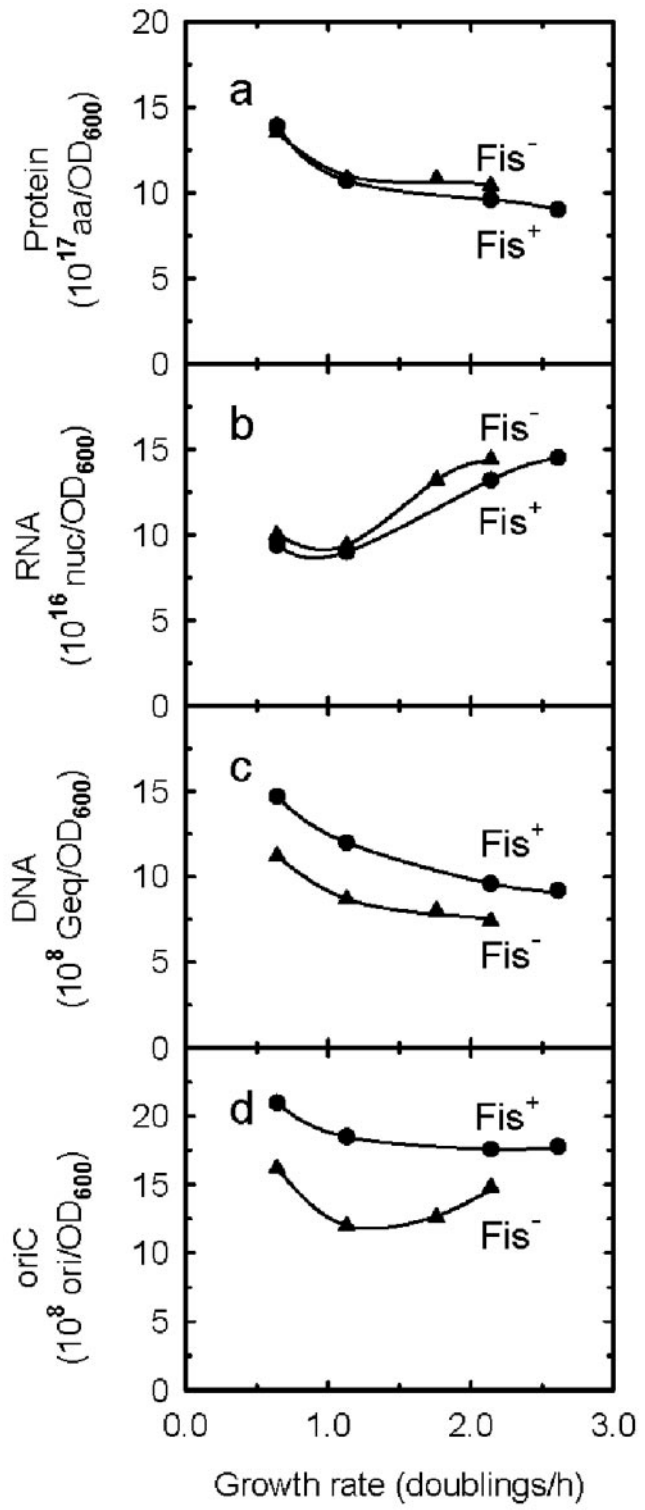

FIG. 4. Protein, RNA, DNA, and *oriC* content in exponential cultures. The amounts of protein, RNA, and DNA and the number of replication origins (*oriC*) per mass unit of bacterial culture (OD_{600}) in wild-type and Fis-deficient *E. coli* strains as a function of growth rate are shown in panels a to d, respectively (data from reference 142). The cultures were grown in glycerol minimal, glucose minimal, glucose amino acids, and LB media (with increasing growth rate; see the text for details).

sions from RNA, DNA, and *oriC* per OD_{600} (Fig. 4b to d) as follows:

(i) The number of *rrn* transcripts per OD_{600} of culture mass, r/OD_{600} = $RNA/OD_{600} \times 0.98 \times 0.86/4{,}566$, where RNA/OD_{600} was taken from Fig. 4b, 0.98 is the fraction of total RNA that is stable RNA (about 2% is mRNA), 0.86 is the fraction of total stable RNA that is rRNA (14% is tRNA), and 4,566 is the number of rRNA nucleotides in the 16S, 23S, and 5S transcripts stemming from one transcription of an *rrn* operon (see reference 16 for more explanations).

(ii) The number of *rrn* transcripts initiated per minute per OD_{600}, $(dr/dt)/OD_{600} = r/OD_{600} \times \ln 2/\tau$, where τ is the culture doubling time (in minutes) (equation 1 above).

(iii) The number of *rrn* transcripts initiated per minute per number of *oriC* copies, $(dr/dt)/ori = (dr/dt)/OD_{600}/oriC/OD_{600}$, where $oriC/OD_{600}$ is taken from Fig. 4d.

(iv) The number of *rrn* transcripts initiated per minute per *rrn* operon, $(dr/dt)/rrn = [(dr/dt)/oriC]/(rrn/oriC)$. Here *rrn*/*oriC* is the number of *rrn* operons per *oriC*, which is obtained from the DNA replication velocity, the culture doubling time, and the map locations of the seven *rrn* operons on the *E. coli* chromosome, as explained in Table 1.

The final result of these conversions is shown in Fig. 5d. The transcriptional activity of an average *rrn* operon is seen to increase in both Fis-proficient and Fis-deficient strains from about 10 transcripts initiated per minute during slow growth in glycerol minimal medium to almost 90 transcripts per minute during fast growth in LB medium.

The Fis paradox. An unexpected result seen in Fig. 5d is that the *rrn* gene activity at a given growth rate is higher in the absence than in the presence of Fis. It is known that Fis stimulates transcription from *rrn* P1 promoters (48, 91, 93, 105, 133). How then can the results in Fig. 5d be correct?

To understand this, it should first be noticed that, in rich media, bacteria without Fis grow more slowly than bacteria with Fis. Therefore, what looks in Fig. 5d like a stimulation of *rrn* gene activity in the absence of Fis at a given growth rate (*y* arrow) should be viewed as an unchanged *rrn* gene activity at a reduced growth rate (*x* arrow) (92). The question then becomes why *rrn* gene activity is unchanged by the removal of Fis and not reduced, as was expected. There are several reasons for this. (i) Due to a reduced DNA concentration (compare DNA/OD_{600} for the two strains in Fig. 4c), the free RNA polymerase concentration in Fis-deficient bacteria is increased, and this increased free RNA polymerase concentration partly compensates for the lack of Fis stimulation of P1. (ii) Due to control of the ppGpp synthetase activity, Fis-deficient strains accumulate less ppGpp (see Fig. 9a below); this stimulates expression from *rrn* P1 promoters. (iii) Finally, Fig. 5d shows transcription from both promoters of *rrn* operons; generally the activity of the downstream P2 promoter is reduced by the activity of the upstream P1 promoter by an effect known as promoter occlusion (142). When P1 activity is reduced due to the absence of Fis, P2 activity increases due to the reduced promoter occlusion. Together these three effects, i.e., increased free RNA polymerase, decreased inhibition by ppGpp, and reduced P2 promoter occlusion, completely compensate for the lack of Fis stimulation of P1 with respect to *rrn* operon activity.

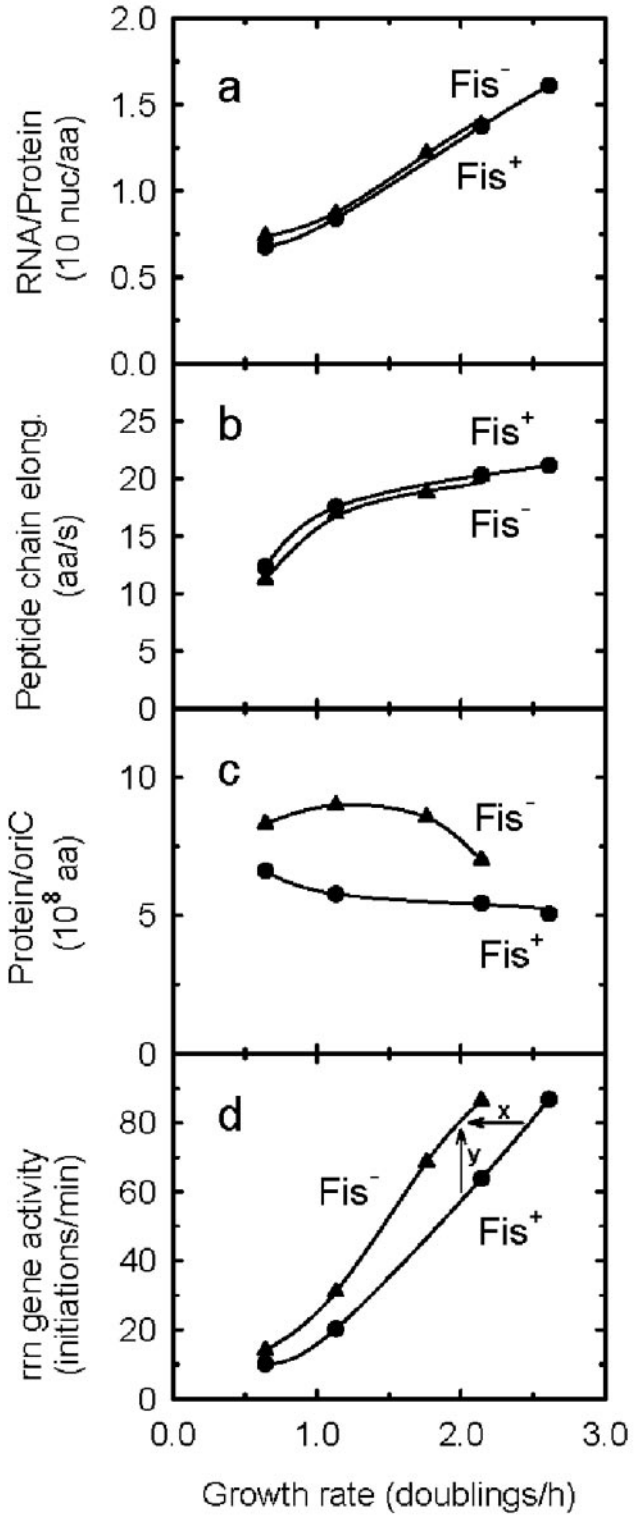

FIG. 5. Activity of *rrn* operons. Ratio RNA per protein, peptide chain elongation rate, protein per *oriC* (initiation mass [36]), and rate of transcription per average *rrn* operon in wild-type and Fis-deficient *E. coli* strains as a function of growth rate are shown in panels a to d, respectively (data from reference 142). This figure is an evaluation of the data in Fig. 4; see the text for details. The peptide chain elongation rate is related to ribosome efficiency (see equation 2) by the factor 0.8 (the fraction of ribosomes that are engaged in translation; the remaining fraction, 0.2, are either ribosome assembly intermediates or ribosomes between rounds of translation, as discussed in reference 16). In panel d, the vertical arrow (*y*) shows the apparent stimulation in *rrn* gene activity at a given growth rate resulting from the absence of Fis; the horizontal arrow (*x*) shows the reduction in growth rate at a constant *rrn* gene activity caused by the absence of Fis.

TABLE 1. Map locations and relative frequencies of the seven *rrn* operons in *E. coli* growing exponentially at 1.0 and at 2.5 doublings/h

Gene	Map units	Distance *m*[a]	*rrn* genes/*oriC*[b]	
			$\mu = 1.0$	$\mu = 2.5$
rrnA	87	0.06	0.96	0.93
rrnB	90	0.11	0.93	0.88
rrnC	85	0.02	0.99	0.98
rrnD	72	0.24	0.85	0.76
rrnE	91	0.13	0.91	0.86
rrnF	57	0.54	0.69	0.54
rrnH	5	0.42	0.75	0.62
All 7 *rrn*/*ori*			6.1	5.6

[a] m = relative distance of *rrn* operon from *oriC* as a fraction of the maximum distance (= length of half chromosome, from *oriC* to *terC*), set at 1.0.

[b] Number (X) of copies of *rrn* operons per *oriC*, $X/oriC = 2^{-mC/\tau}$ (13) where C = time to replicate the chromosome from replication origin to terminus. At $\mu = 1.0$ doublings/h, $C = 60$ min; at $\mu = 2.5$ doublings/h, $C = 40$ min (13). τ = doubling time = $60/\mu$.

If the absence of Fis does not reduce the activity of *rrn* operons, one might ask why the growth rate is reduced in Fis-deficient strains. The answer is that the total rate of rRNA synthesis is reduced, despite unchanged transcription per *rrn* operon, because of the decreased number of *rrn* operons per mass unit of culture. Thus, paradoxically, Fis is found to stimulate the growth rate because it stimulates DNA replication, not because it stimulates rRNA synthesis. A further analysis of this paradox is clearly required but is beyond the scope of this review.

Relative Expression from *rrn* P1 and P2 Promoters

To determine the expression from the *rrnB* P1 and P2 promoters relative to expression from the tandem P1-P2 promoters of *rrnB*, the two isolated P1 and P2 promoters and the tandem P1-P2 promoters were fused to a *lacZ* reporter gene on a promoter cloning plasmid in which the promoter-*lacZ* region is flanked by sections of the chromosomal *mal* operon (141). This allows one to recombine the promoter-*lacZ* fusions into the *mal* operon of the chromosome. This region is close to and in the same orientation as *rrnB* in its normal location, so that the direction of transcription coincides with the direction of chromosome replication. In these gene fusions, the promoters are separated from the *lacZ* translation start by a 1-kb insertion of DNA from phage λ. This was important because it has been observed that the translation of *lacZ* is frequently affected by structures in the 5′-terminal mRNA immediately downstream of the transcription start and upstream of the ribosome-binding site for *lacZ* (76). In the *rrnB* promoter fusions used (Fig. 6), the region immediately upstream of the *lacZ* translation start was always the same λ DNA, independent of the cloned promoter, so that promoter effects on *lacZ* translation were absent (76). In addition, all fusions contained the *rrn* antitermination sequences (3, 75).

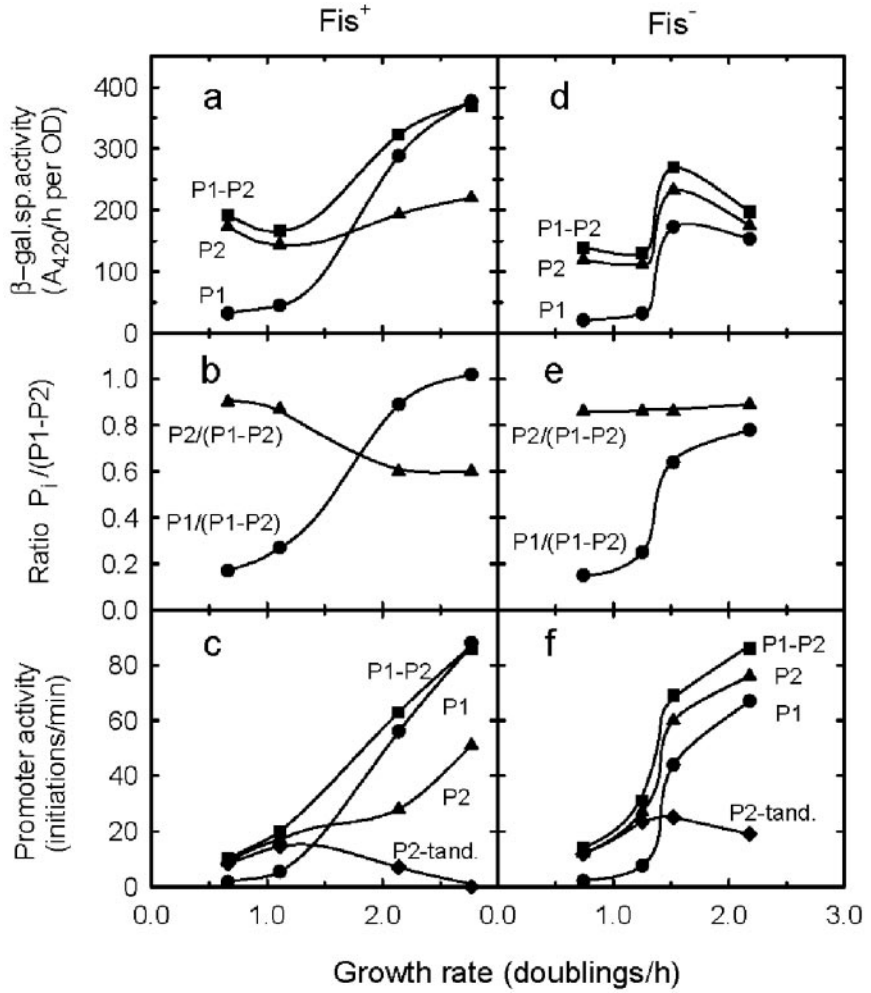

FIG. 6. Activity of the P1, P2, and P1-P2 promoters. (a) and (d) β-galactosidase specific activity expressed from *rrnB* P1, P2, and the tandem P1-P2 promoters in wild-type and Fis-deficient *E. coli* strains as a function of growth rate (data from reference 142). (b and e) Ratios of β-galactosidase specific activities expressed from *rrnB* P1 and P1-P2 and from P2 and P1-P2, respectively. (c and f) Absolute activities of the tandem P1-P2 promoters (from Fig. 5d), of the isolated P1 and P2 promoters, and of the P2 promoter when it is in tandem, downstream of P1. The points are plotted at the average growth rate for the three cultures used in the *fis*$^+$ and Δ*fis* strain backgrounds, i.e., carrying the P1-*lacZ*, P2-*lacZ*, and P1-P2-*lacZ* fusions, respectively. In panels c and f, the absolute activities for the P1-P2 strains (square symbols) taken from Fig. 5d are plotted at the (slightly different) growth rates observed for the cultures used for the β-galactosidase determinations in panels a and d, respectively (see the text for further explanations).

Figure 6 panels a and d show the β-galactosidase activities expressed at different growth rates from the separated *rrnB* P1 and P2 promoters and from the tandem P1-P2 promoters for two isogenic *fis*$^+$ (left panels) and Δ*fis* (right panels) strains. In *fis*$^+$ bacteria, the expression from P1 increases with growth rate (Fig. 6a, circles), whereas the enzyme specific activity expressed from P2 is nearly constant (Fig. 6a, triangles). In the past, such *lacZ* expression data from *rrn* P1 and P2 promoters have been interpreted as an indication that the P1 promoters are controlled by the growth rate, whereas the P2 promoters lack a growth rate-dependent control (49, 61). However, as was shown above (Fig. 3), unambiguous conclusions about promoter control cannot be drawn from enzyme expression data at different growth rates without further considerations.

Figures 6b and e show P1 and P2 expression relative to expression from the reference P1-P2. Again, these ratios cannot be interpreted in terms of promoter activities or control; they are but a part of the total information that is required to estimate the absolute promoter activities.

Use of translation and transcription assays. The results from transcriptional or translational assays of gene expressions from endogenously controlled promoters observed at different growth rates show large quantitative discrepancies as a result of the different reference units used for the assays (Fig. 3). However, the expression ratios P1/(P1-P2) and P2/(P1-P2) are the same when they are obtained from either transcription (hybridization) assays by measuring *lacZ* mRNA or from β-galactosidase activity measurements, as long as the samples for preparing RNA for hybridization assays are taken from the same cultures that are used for the enzyme activity measurements (76). This is because all effects of the different reference units cancel in the ratios. Therefore, the following determination of absolute promoter activities is independent of the type of assay (mRNA or enzyme activity) used for obtaining the expression ratios.

Absolute Transcriptional Activities of *rrnB* P1 and P2

As was explained in the Rationale of the Method and in equation (11), the transcriptional activity of the isolated *rrn* P1 and P2 promoters is calculated as the product of the absolute activity of the *rrn* P1-P2 tandem reference promoter (Fig. 5d) times the relative P1/(P1-P2) and P2/(P1-P2) expression ratios, respectively (Fig. 6b and e). When this is done, it is seen that the activities of both isolated *rrn* promoters increase with increasing growth rate (Fig. 6c and f, circles and triangles). In the presence of Fis, P1 activity increases almost 50-fold in the range of growth rates studied, from about 2 to almost 90 initiations/minute, whereas the activity of the isolated P2 promoter starts out higher, at about 10 initiations/minute, but increases less, to only about 50 initiations/minute. In the absence of Fis, the P1 and P2 activities at low growth rates are similar to the activities observed in the presence of Fis. However, at high growth rates, P1 activity rises less because of the lack of Fis stimulation, and P2 activity rises more because of the increased free RNA polymerase concentration (see below).

The rRNA chain elongation rate sets a maximum limit of about 110 initiations/minute for the rate of transcript initiation at an rRNA promoter (see the section Transcript Initiation under In Vivo Conditions above and the section Mathematical Modeling of *rrn* Transcript Initiation below). The initiation rate approaches 80% of this maximal value at high growth rates, and there is little indication of promoter saturation (Fig. 6c and f). For this reason we have previously concluded that V_{max} for rRNA promoters is limited by the chain elongation steps that occur after transcript initiation and are required for promoter clearance and regeneration, so that V_{max} is assumed to be close to 110 initiations/minute (142, 143).

There are several options to explain the varying *rrn* promoter activities. For example, Barker et al. (10, 11) proposed that *rrn* promoters are saturated, so that changes in the free RNA polymerase concentration do not contribute to the changing *rrn* promoter activities, and therefore both promoters must be under some form of specific growth medium-dependent control. To distinguish between this and other possibilities, it is necessary to obtain information about the concentration of free RNA polymerase in the bacterial cytoplasm. How this is done is explained in the sections below.

***rrn* P2 promoter occlusion.** In addition to the activity of the P2 promoter when it is isolated from P1, Fig. 6c and f also show the activity of the P2 promoter when it is in tandem with and downstream of the P1 promoter (P2-tand; diamonds). These curves were obtained as the difference in activities P1-P2 (tandem) minus P1 (separated). At high growth rates, the activity of the tandem P2 promoter is seen to be reduced in comparison to the activity of the separated P2 promoter. This reduction in activity when P2 is located downstream of an active P1 promoter is known as promoter occlusion. The probability that the P2 promoter is occluded can be predicted by a theory that takes into account the transcriptional activity of the P1 promoter, the length of DNA required for promoter binding, and the length of DNA covered by a transcribing RNA polymerase. The theoretical prediction agrees well with the observed extent of occlusion (142).

Free RNA Polymerase Concentration in the Bacterial Cytoplasm

In the preceding section, it was described how the absolute activities of the *rrn* P1 and P2 promoters were found. These determinations form the basis for the following evaluation in terms of the growth rate-dependent control of rRNA synthesis. For this evaluation, it is first necessary to obtain information about the growth rate-dependent changes in the concentration of free RNA polymerase in the cells.

Methods for determination of free RNA polymerase concentration. Three methods are available to determine the free RNA polymerase concentration in the bacterial cytoplasm.

(i) The most direct way consists of determination of the concentration of total cytoplasmic RNA polymerase, which consists of free functional and immature nonfunctional RNA polymerase. The total free RNA polymerase has been estimated from the amount of RNA polymerase subunits in DNA-free minicells of certain mutant bacterial strains (122); the immature RNA polymerase can be found from the maturation time of RNA polymerase, observed as the time lag between the synthesis of RNA polymerase subunits and their appearance in the nucleoid (109). By subtracting the immature from the total cytoplasmic polymerase, it was estimated that $[R_f]$ is 1.2 μM for bacteria growing at 2.5 doublings/h in rich media (19). This value corresponds to 9% of the total RNA polymerase in the cell and is consistent with other available data. However, this estimate was based on experiments done in different laboratories with nonisogenic strains, and it is too complex to be readily applicable to different growth conditions and strains.

(ii) Another method consists of probing the free functional RNA polymerase with a suitable constitutive, unsaturated promoter that is linked to a reporter sequence. For this purpose we have chosen the *rrnB* P2 promoter linked to *lacZ*. The K_m for RNA polymerase binding to this promoter under in vivo conditions is not known, but one can obtain relative values, $[R_f]_{rel}$, with this method, i.e., the free RNA polymerase concentration in the cytoplasm relative to the free RNA polymerase concentration at which the *rrn* P2 promoter shows half-maximal activity (set equal to one concentration unit). By applying the Michaelis-Menten relationship (equation 4 above) and setting V_{max} to 110 initiations/minute (see above), $[R_f]_{rel}$ (i.e., the quotient $[R_f]/K_m$) is found from the *rrn* P2 promoter activity, V_{P2}:

$$[R_f]_{rel} = 1/[(110/V_{P2}) - 1] \quad (12)$$

With the P2 activity data (Fig. 6c and f, triangles), $[R_f]_{rel}$ was found to increase with increasing growth rate, as illustrated in Fig. 1a above.

In contrast to these findings, it has been proposed recently that the free RNA polymerase concentration should decrease with increasing growth rate as a consequence of the increased induction of rRNA transcription at higher growth rates and the resulting sequestering of large numbers of RNA polymerase molecules on the *rrn* operons (10, 11). However, the results of a computer simulation of global transcriptional activities at different growth rates (19) confirmed that the free RNA polymerase concentration can be expected to increase, not decrease, with increasing growth rate (see the following paragraph).

(iii) The concentration of free RNA polymerase can also be determined from the observable concentration of total RNA polymerase, the concentration and properties of all promoters in the cell, and their associated transcript lengths and transcription velocities. Since kinetic parameters are unknown for most mRNA promoters, this method can only be applied to a simplified model by grouping all bacterial promoters into a small number of different classes with assumed average promoter properties. These average classes are based on the observed properties of certain promoters that are assumed to be typical of their class. The results of such modeling of global transcription in *E. coli* showed that free RNA polymerase is indeed a small fraction of the total RNA polymerase concentration and that this fraction increases with increasing growth rate as a result of the increased synthesis of total RNA polymerase (120, 121) and increased repression of mRNA genes by nutrients present in rich growth media (19).

Constitutivity of the *rrn* P2 promoter. Before the method to find $[R_f]_{rel}$, described in the preceding section ii, was applied to observed *rrn* P2 promoter activities, the question as to whether the P2 promoter is constitutive had to be answered. The answer to this question is controversial. Recently, it was reported that "*rrnB* P2 is regulated: it displays clear responses to amino acid availability (stringent control), rRNA gene dose (feedback control) and changes in growth rate (growth rate-dependent control)" (87). However, this question depends, first, on the definition of control. In the definition used by those authors, changes in gene expression caused by changes in free RNA polymerase concentrations would be included in the term control, whereas we exclude such effects. Second, those authors define changes in enzyme expression from a given promoter as changes in gene activities, which is incorrect for changing growth conditions (see Fig. 3 above).

These issues have been addressed previously in detail (143). In summary, the answer is that no control or factor binding sites are known for the *rrn* P2 promoter and that, under various conditions, P2 behaves in every respect like a constitutive, unsaturated promoter. For example, at low growth rates, P2 promoter activity increases with growth rate in parallel with the activity of a number of other promoters that are generally assumed to be constitutive, including the β-lactamase promoter P_{bla} and the P_L promoter of phage λ (78). Furthermore, changes in DNA concentration, e.g., in replication-defective mutants (25), affect the concentration of free RNA polymerase (see Fig. 2) and change P2 promoter activity in exactly the way that would be expected for unsaturated, uncontrolled promoters. An alternative hypothesis is that all bacterial promoters previously considered constitutive are in fact subject to a nonspecific and growth medium-dependent control that results in exactly the same activity changes as would be expected for constitutive promoters subject to varying concentrations of free RNA polymerase. This is logically possible but appears unlikely.

Based on the observation that *rrn* P2 promoter activity is reduced during the stringent response, it has been concluded previously that *rrn* P2 promoters are subject to stringent control (45). However, the authors did not consider the effects of free RNA polymerase. The severely reduced concentration of free RNA polymerase associated with the stringent response (106) affects all unsaturated promoters, constitutive and regulated. This effect needs to be distinguished from that of ppGpp accumulation during the stringent response, which reduces the strength of the P1 promoter but not that of the constitutive P2 promoter.

The most direct experimental support for the constitutivity of the *rrn* P2 promoter comes from experiments in which the nutritional quality of the growth medium was improved minimally by the addition of single amino acids to cultures growing in minimal medium. In such single-amino-acid upshifts, transcription from *rrn* P1 promoters increases up to 100% depending on the particular amino acid added to the medium, but the activity of the P2 promoter and the growth rate remain essentially unchanged (X. Zhang et al., unpublished data). Since the rate of total transcription increases by only a few percentage points in such single-amino-acid upshifts, no significant change in the free RNA polymerase concentration, and therefore no change in the activity of constitutive promoters, is to be expected. This agrees with the observed absence of stimulation for P2 by the single-amino-acid upshifts despite the clear stimulation of P1.

rrn P1 Promoter Strength at Different Growth Rates

With relative values for $[R_f]$ obtained from the *rrn* P2 activity, it becomes possible to separate the effects of specific control factors on the activity of the *rrn* P1 promoter from the nonspecific effects of changing free RNA polymerase concentrations. For this purpose, the Michaelis-Menten relationship (equation 4) was applied to P1 activity, V_{P1}. Since equation 4 has four parameters and only two are known, i.e., V_{P1} and $[R_f]_{rel}$, it is not possible to find separate values for V_{max} and K_m, but their ratio V_{max}/K_m, which is the relative P1 promoter strength (142), can be estimated. The resulting promoter strength values for ppGpp-deficient (Δ*relA* Δ*spoT*) and ppGpp-proficient (*relA*$^+$ *spoT*$^+$) bacteria (from reference 143) and for Fis-deficient (Δ*fis*) and Fis-proficient (*fis*$^+$) strains are illustrated (Fig. 7 and 8).

In ppGpp-proficient strains, relative P1 promoter strength increases with increasing growth rate to a highest value corresponding to the same level found in ppGpp-deficient strains at any growth rate. This value is about three times higher in the presence than in the absence of Fis. Thus, in wild-type (ppGpp- and Fis-proficient) strains, P1 promoter strength increases about 15-fold for a 4-fold increase in growth rate, whereas in the absence of Fis, the growth rate-dependent increase in the P1 promoter strength is only about 5-fold. In the absence of ppGpp, P1 promoter strength becomes constant, i.e., the P1 promoter behaves like a constitutive promoter whose strength depends only on the presence or absence of Fis.

Control of rRNA Synthesis by ppGpp and Fis

The results from the genetic deletion data in Fig. 7 and 8 show that the products of the genes *relA*, *spoT*, and *fis* are causally involved in the growth rate-dependent control of *rrn* P1 promoter strength. The *relA* and *spoT* products are ppGpp synthetases, and *fis* expression is known to be ppGpp dependent (7, 93, 94). Therefore, we measured basal levels of ppGpp accumulating at different growth rates in ppGpp-proficient strains. The results were expressed in molar units from the UV absorbance of the ppGpp peak after chromatographic separa-

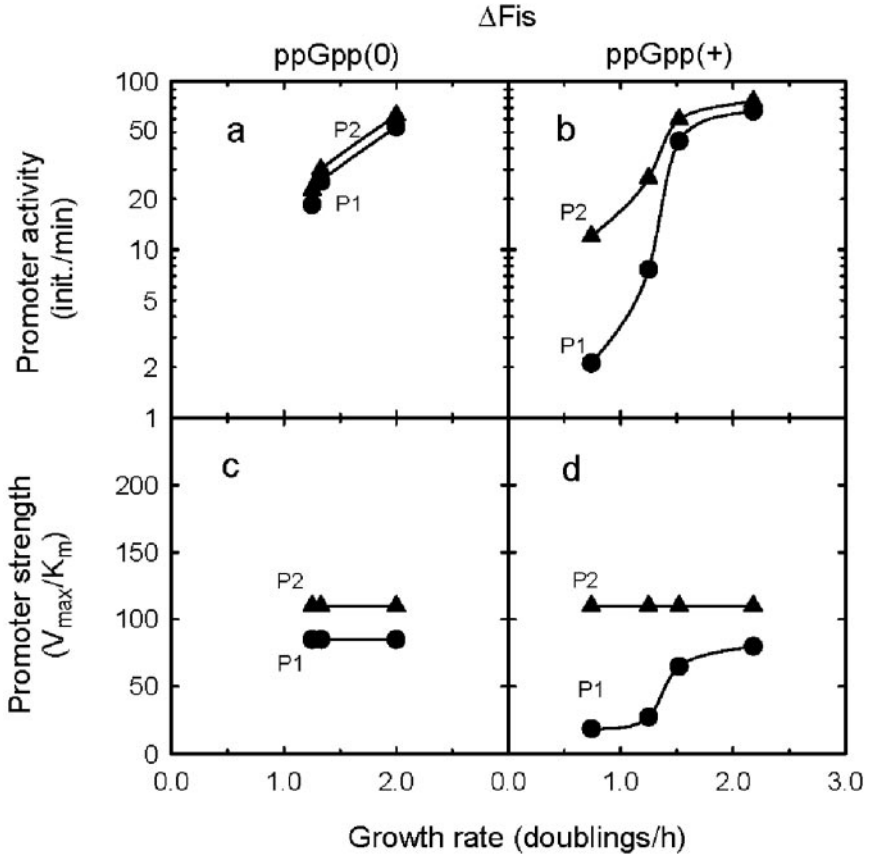

FIG. 7. Growth rate dependence of transcriptional activities and strength of the *rrnB* P1 and P2 promoters in the absence of Fis. Data on transcriptional activity (upper panels) and promoter strength (V_{max}/K_m, lower panels) for *rrnB* P1 (●) and P2 (▲) in Δ*fis* strains in the absence (left panels) and presence (right panels) of ppGpp (data from reference 143). The P2 promoter strength values (▲) in the lower panels are assumed to be always constant (K_m = 1.0, V_{max} = 110, V_{max}/K_m = 110; see the text).

tion from other nucleotides by high-pressure liquid chromatography (82). The alkali method used for this purpose completely lyses the bacteria, so that nucleotides are 100% released (82) (see also the discussion above in the section on NTP models in Historical Overview). The level of cytoplasmic ppGpp decreases with increasing growth rate in both Fis-proficient and Fis-deficient bacteria (Fig. 9a) (143). However, in Fis-deficient bacteria, the level of ppGpp decreases faster in comparison to Fis-proficient bacteria (Fig. 9a). This difference reflects the control of ppGpp accumulation by the ppGpp synthetase/hydrolase activities of the *spoT* product. In this manner, the decreasing strength of the *rrn* P1 promoters due to the absence of Fis is partly compensated for by the increasing strength due to the reduced level of ppGpp.

In Fig. 9b, the results of the ppGpp determinations in Fig. 9a are combined with the promoter strength determinations in Fig. 7 and 8. P1 promoter strength is seen to decrease with increasing cytoplasmic levels of ppGpp. In the absence of ppGpp or at low concentrations of ppGpp during growth in rich media, Fis stimulates the P1 promoter threefold (Fig. 9b). The extent of this stimulation decreases with the increasing level of ppGpp, until it is totally absent at high levels of ppGpp during slow growth in poor media. This can be explained by the negative control of Fis synthesis by ppGpp, which is expected to prevent Fis activation of P1 during slow bacterial growth (7, 93, 94).

During fast growth at very low levels of ppGpp in ppGpp-proficient strains, P1 promoter strength has the same highest value as at any growth rate in the total absence of ppGpp in ppGpp-deficient strains (compare circles in panels c and d of Fig. 7 and 8). These observations, taken together with those in Fig. 9, clearly implicate ppGpp as a negative effector in the control of P1 promoter strength. However, the results do not indicate if the control is direct or indirect. Rather than acting directly by reducing the RNA polymerase-P1 promoter interaction, ppGpp could positively control the synthesis of an inhibitory factor that binds to *rrn* P1 promoters. A potential candidate for such an inhibitor is H-NS, a factor that appears to inhibit transcription from the P1 promoter in vitro (2, 127, 128). However, no difference in the in vivo activity of P1 was found between *hns*$^+$ and *hns* strains (Fig. 4b of reference 1).

The observation that ppGpp binds to RNA polymerase (24, 102, 129) suggests that this binding directly affects transcript initiation at the promoters of genes assumed to be regulated by ppGpp, including *rrn* P1, *fis*, and genes of additional unidentified factors that might affect P1 promoter strength. All such effects, direct and indirect, on P1 promoter strength are included in the ppGpp effects seen in Fig. 9b.

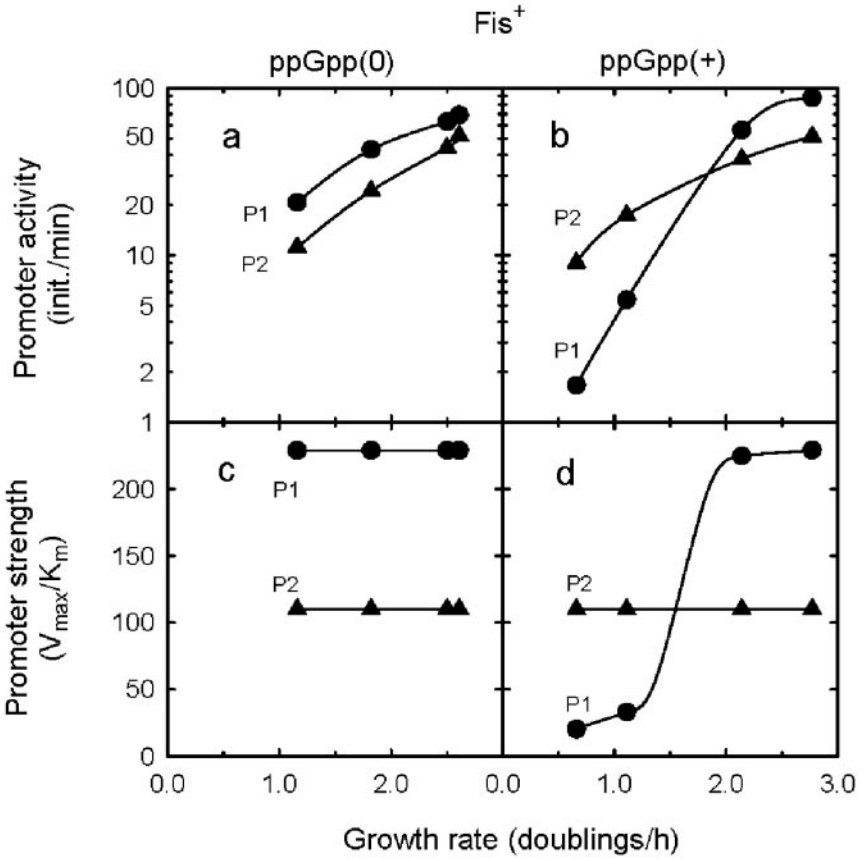

FIG. 8. Growth rate dependence of transcriptional activities and strength of the *rrnB* P1 and P2 promoters in the presence of Fis. Data on transcriptional activity (upper panels) and promoter strength (V_{max}/K_m, lower panels) for *rrnB* P1 (●) and P2 (▲) in *fis*$^+$ strains in the absence (left panels) and presence (right panels) of ppGpp (data from reference 143). The P2 promoter strength values (▲) in the lower panels are assumed to be always constant (K_m = 1.0, V_{max} = 110, V_{max}/K_m = 110; see the text).

The question remains how it can be proved that ppGpp controls *rrn* P1 promoter strength directly by changing the interaction of RNA polymerase with the P1 promoter and not indirectly by controlling the synthesis or activity of another factor that binds to the P1 promoter region. It might appear that such a proof can only come from in vitro experiments. Numerous research groups have shown in vitro inhibition of P1 activity by ppGpp (10, 50, 53, 65, 66, 98). However, depending on the assay conditions (including the degree of superhelicity of the templates), the in vitro results have differed quantitatively from in vivo observations, presumably because complex in vivo conditions cannot yet be reproduced in vitro. For example, a recent study (10) found that ppGpp inhibits the formation of the open complex at P1 (see Historical Overview above, new ppGpp model), but in that study the V_{max} for P1 was at least 100 times lower than was observed in vivo (143). Therefore, interpretation of existing in vitro data relating to the in vivo effects of ppGpp at the P1 promoter remains problematic.

A potential solution to this dilemma may already exist. As mentioned earlier (Historical Overview), ppGpp-dependent in vivo inhibition of the P1 promoter can be abolished by a base change in the GCGC discriminator region bordering the −10 TATAAT region of the *rrn* P1 promoter (139, 140). Since this region is known to be contacted only by the RNA polymerase, it is very likely that *rrn* P1 promoter strength is subject to a direct inhibition resulting from ppGpp binding to the RNA polymerase. Once this result is confirmed and becomes more firmly established, the further question about the molecular mechanism of P1 inhibition by ppGpp can be addressed (see Mathematical Modeling of the Control of *rrn* Transcript Initiation below for a step in that direction).

If we assume that ppGpp is a direct effector of transcript initiation at *rrn* P1, the half-maximal reduction in P1 promoter strength by ppGpp occurs at a level of ppGpp corresponding to approximately 20 pmol/OD_{460} (Fig. 9b). Previously, it was found that the fraction of total RNA (mRNA + tRNA + rRNA) synthesis that is stable RNA (tRNA + rRNA), r_s/r_t, decreases with increasing cytoplasmic level of ppGpp from a value greater than 0.9 at near zero levels of ppGpp to a minimum value of 0.25 at high levels of ppGpp (17, 107). The 25% residual rRNA synthesized at high levels of ppGpp originates predominantly at the *rrn* P2 promoters that are not specifically inhibited or controlled by ppGpp (141). In those previous experiments, the half-maximal reduction in r_s/r_t occurred at 20 pmol/OD_{460} (8, 17, 107), i.e., the same value as found for the half-maximal reduction in P1 promoter strength (Fig. 9b). The value of 20 pmol/OD_{460} corresponds to a molar concentration of about 50 μM (143). We suggest that RNA polymerase is half-saturated with ppGpp at this concentration, which then would be equal to the dissociation constant (K_d) for ppGpp

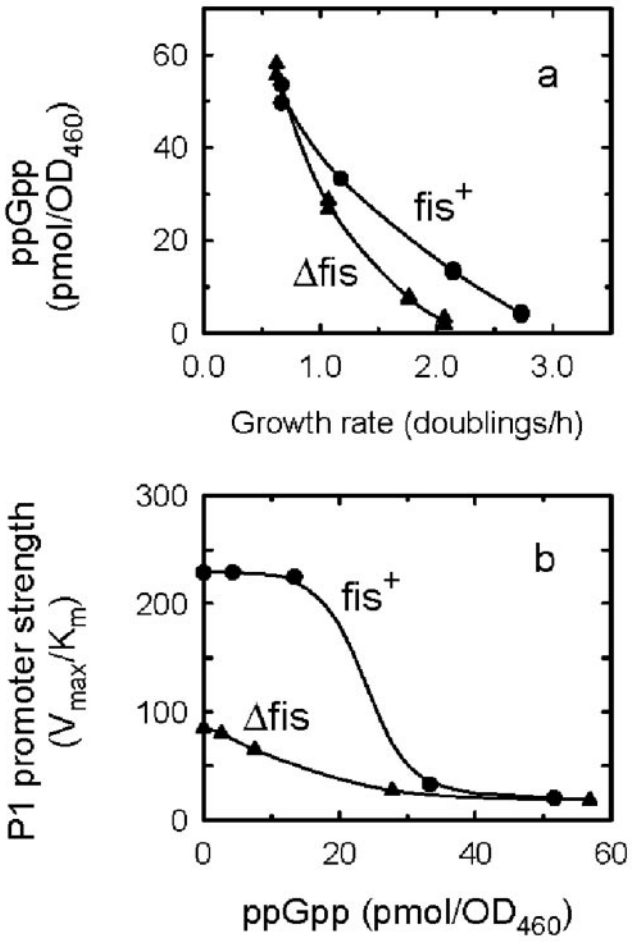

FIG. 9. Effect of ppGpp accumulation on *rrnB* P1 promoter strength. (a) The accumulation of ppGpp was measured during exponential growth at different rates in the Fis-proficient (●) and Fis-deficient (▲) strains (data from reference 143). The results of two determinations from the same preparation of nucleotides are shown. (b) The promoter strength (V_{max}/K_m) of *rrnB* P1 as a function of ppGpp in Fis-proficient (●) and Fis-deficient (▲) strains was obtained by combining the data in panel a with the data in Fig. 7d and 8d (circles), respectively.

binding to RNA polymerase. Bacteria growing in glucose minimal medium contain approximately 20 pmol/OD_{460} (Fig. 9a); therefore, we suggest that under those conditions, about half of all RNA polymerase molecules have ppGpp bound.

To summarize our conclusions about the control of rRNA synthesis: *rrn* P1 activity increases about 50-fold when the growth rate of a wild-type strain increases about fourfold (Fig. 8b) as a result of the combined effects of decreasing inhibition by ppGpp, increasing stimulation by Fis, and increasing concentration of free RNA polymerase. In contrast, transcription coming from the (tandem) P2 promoters of *rrn* operons decreases to near zero at the higher end of the growth rate range as a result of increasing P2 promoter occlusion by increasing P1 activity upstream of P2 (Fig. 6c, diamonds).

Mathematical Modeling of the Control of *rrn* Transcript Initiation

Reactions involved in transcript initiation. In order to model the process of *rrn* transcript initiation and the effects of ppGpp and Fis on it, we derive in the following the Michaelis-Menten relationship describing the activity of a given promoter, V, as a function of its kinetic constants, V_{max} and K_m, and of the free RNA polymerase concentration, $[R_f]$, from the underlying rate constants of the subreactions involved in transcript initiation. For easier reference, we repeat the reaction scheme for transcript initiation (see Theory above) and the definitions of its rate constants:

$$\mathrm{R + P} \underset{k_{-\mathrm{I}}}{\overset{k_{\mathrm{I}}}{\leftrightarrow}} \mathrm{RP}_{c1} \underset{k_{-\mathrm{II}}}{\overset{k_{\mathrm{II}}}{\leftrightarrow}} \mathrm{RP}_{o2} \underset{k_{-\mathrm{III}}}{\overset{k_{\mathrm{III}}}{\leftrightarrow}} \quad \text{(reversible)}$$

$$\mathrm{RP}_{\mathrm{init}(1,m)} \overset{k_{\mathrm{IV}}}{\rightarrow} \mathrm{TC}_{(m+1,\,n)} \overset{+\sigma \quad k_{\mathrm{V}}}{\rightarrow} \mathrm{TC}_{(n+1)} + \mathrm{P} \quad \text{(irreversible)}$$

In this scheme, R is the free RNA polymerase holoenzyme, P is the free promoter, RP_{c1} is the closed complex, RP_{o2} is the open complex, $RP_{init(1,\,m)}$ is the initiation complex, including abortive initiations, when the transcript has a length of less than m nucleotides ($m = 10$; it might be more appropriate to define the initiation complex as $RP_{init(2,\,m)}$, i.e., after the formation of the first phophodiester bond that increases the stability of the complex), $TC_{(m\,+\,1,\,n)}$ is the transcription complex after the release of σ at m nucleotides when the transition from initiation to elongation is complete and the transcript has a length of between $m + 1$ and n nucleotides ($n = 50$), and $TC_{(n\,+\,1)}$ is the transcription complex after promoter regeneration when the polymerase has moved $n + 1$ bp (i.e., 51 bp) away from the promoter.

The rate constants associated with these reactions are defined by their reciprocals as follows: $1/(k_{\mathrm{I}}[R_f])$ is the average time required to bind once to the promoter (or $1/k_{\mathrm{I}}$ is the average time required to bind once to the promoter at $[R_f] =$ 1 unit; in the following, 1 unit is assumed to be 1 μM); $1/k_{\mathrm{II}}$ is the average time to go once from RP_{c1} to RP_{o2}; $1/k_{\mathrm{III}}$ is the average time to go once from RP_{o2} to $RP_{init(1)}$; $1/k_{\mathrm{IV}}$ is the time required to go from $RP_{init(1)}$ to $TC_{(m\,+\,1)}$; $1/k_{\mathrm{V}}$ is the time required to sufficiently elongate the transcript to regenerate a free promoter; $1/k_{-\mathrm{I}}$ is the average time the RNA polymerase remains in RP_{c1} before dissociating; $1/k_{-\mathrm{II}}$ is the average time the RNA polymerase remains in RP_{o2} before reverting to RP_{c1}; and $1/k_{-\mathrm{III}}$ is the average time the RNA polymerase remains in $RP_{init(1)}$ before reverting to RP_{o2}.

Rate of transcript initiation. The average time between two transcript initiations, τ_{ini}, equals the sum of the times required for all five reactions involved in transcript initiation:

$$\tau_{ini} = \tau_{c1} + \tau_{o2} + \tau_{init} + \tau_{TC10} + \tau_{TC50} \qquad \text{(A1)}$$

These times are defined as follows: τ_{c1} is the average time required to form RP_{c1} per initiation, including repeats due to reversibility; this time equals the average time per initiation that the promoter stays free or the average time required for the polymerase to bind [τ_{c1} equals t_b in equation 6 and in Fig. 1b and c above). τ_{o2} is the average time per initiation required to form RP_{o2} from RP_{c1}, including repeats; this time equals the average time per initiation that the promoter stays in the closed complex. τ_{init} is the average time per initiation required to form $RP_{init(1)}$ from RP_{o2}, including repeats; this time equals

the average time per initiation that the promoter stays in the open complex. τ_{TC10} is the average time the polymerase stays in $RP_{init(1,m)}$ before the release of σ; and τ_{TC50} is the average time the polymerase stays in $TC_{(m+1,n)}$ after the release of σ. These times equal the product of the time required for one occurrence of the reaction (see definitions of reciprocals of rate constants above), and the number of times, n, the reaction is repeated for one successful initiation:

$$\tau_{c1} = n_{c1}/(k_I \cdot [R_f]) \quad (A2)$$

$$\tau_{o2} = n_{o2}/k_{II} \quad (A3)$$

$$\tau_{init} = n_{init}/k_{III} \quad (A4)$$

$$\tau_{TC10} = 1/k_{IV} \quad (A5)$$

$$\tau_{TC50} = 1/k_V \quad (A6)$$

The number of repeats of the first three (reversible) reactions are defined as follows: n_{c1} is the average number of times the polymerase has to bind per initiation; n_{o2} is the average number of times the open complex has to be formed per initiation; and n_{init} is the average number of times the initiation complex has to be formed per initiation. These numbers of repeats depend on the rate constants as follows:

$$n_{c1} = 1 + (k_{-I}/k_{II}) \cdot n_{o2} \quad (A7)$$

$$n_{o2} = 1 + (k_{-II}/k_{III}) \cdot n_{init} \quad (A8)$$

$$n_{init} = 1 + (k_{-III}/k_{IV}) \quad (A9)$$

For the last two irreversible reactions that involve the transcription complex and RNA chain elongation, this number is 1.0.

Maximum activity, V_{max}. The maximum promoter activity (initiations per minute) at promoter saturation is referred to as V_{max}. Its reciprocal, the minimum average time between transcript initiations, $\tau_{min} = 1/V_{max}$, equals the sum of the four times required for the reactions occurring after the initial binding:

$$\tau_{min} = 1/V_{max} = \tau_{o2} + \tau_{init} + \tau_{TC10} + \tau_{TC50} \quad (A10)$$

or, with the expressions for the τ values above (equations A3 to A6):

$$\tau_{min} = n_{o2}/k_{II} + n_{init}/k_{III} + 1/k_{IV} + 1/k_V \quad (A10a)$$

This equation corresponds to equation 7 above. τ_{min} also equals the average time per initiation that the promoter is occupied by an RNA polymerase.

Free RNA polymerase concentration at half-maximal activity, K_m. The concentration of free RNA polymerase at which the promoter activity is half-maximal is referred to as K_m. At this concentration, the promoter is free 50% of the time and occupied by an RNA polymerase 50% of the time. The half-maximal rate is obtained when $\tau_{ini} = 2\tau_{min}$, or when the average total time required for promoter binding, including repeats of binding required for one successful initiation, equals the total average time required for all reactions occurring after the initial binding, including possible repeats of these reactions, i.e., if $\tau_{c1} = \tau_{min}$. Substituting equation A2 in this identity gives

$$\tau_{c1} = n_{c1}/(k_I \cdot [R_f]) = \tau_{min}$$

and resolving for $[R_f]$, we find

$$[R_f] = K_m = n_{c1}/(k_I \cdot \tau_{min}) = (n_{c1}/k_I) \cdot V_{max} \quad (A11)$$

and the promoter strength

$$(V_{max}/K_m) = (k_I/n_{c1}) \quad (A11a)$$

Michaelis-Menten relationship for promoter activity. With equations A1, A2, A10, and A11a, the average time between two initiations becomes

$$\tau_{init} = \tau_{c1} + \tau_{min} = n_{c1}/k_I \cdot 1/[R_f] + \tau_{min}$$
$$= \tau_{min} \cdot [1 + (K_m/[R_f])] \quad (A12)$$

In its reciprocal form, equation A12 represents the standard the Michaelis-Menten relationship

$$1/\tau_{init} = V = V_{max} \cdot 1/[1 + (K_m/[R_f])] \quad (A12a)$$

Rate constants for *rrn* promoters. In the following, the Michaelis-Menten analysis derived above is applied to the *rrn* P1 promoter in the absence of both Fis and ppGpp. Under such conditions, the *rrn* P1 promoter resembles the constitutive *rrn* P2 promoter (see Fig. 7a), for which approximate values for V_{max} = 110 initiations/minute and K_m = 4.35 μM have been estimated (19, 143).

The last two steps in the five-step initiation reaction scheme above require RNA chain elongation until the RNA polymerase has moved 50 bp away from the promoter (i.e., an *rrn* transcript of 50 nucleotides must be synthesized) before a free promoter is regenerated and a new RNA polymerase can bind. The rRNA chain elongation rate has been determined to be 90 nucleotides per second (134). Therefore, the sum (τ_{TC10} + τ_{TC50}) in equation A3 is assumed to equal to 50/90 = 0.55 s. This gives an upper limit for V_{max}: if the times for open complex and initiation complex formation, τ_{o2} and τ_{init}, were negligibly small compared to 0.55 s, then V_{max} would be 1.8 (= 1/0.55) initiations per second, or 108 initiations per minute. Since *rrn* promoter activities of 80 to 90 initiations/minute have been observed without evidence for promoter saturation (Fig. 7b and 8b), we concluded previously that τ_{o2} and τ_{init} are very much smaller than 0.55 s and in the millisecond range, so that V_{max} for *rrn* promoters equals about 110 initiations per minute.

It is also known that the closed and open complexes for *rrn* promoters are extremely unstable, so that the existence of these complexes cannot be demonstrated by DNA footprinting (9). In contrast, the initiation complex with the first two nucleotides of the transcript, $RP_{init(2)}$, is very stable and can be detected by footprinting. With the observed estimate for K_m (4.35 μM), the following set of rate constants for the *rrn* P2 promoter and the *rrn* P1 promoter in the absence of Fis and ppGpp may be assumed: $1/k_I$ = 0.35 s per $[R_f]$, $1/k_{II}$ = 0.002 s, $1/k_{III}$ = 0.002 s, $1/k_{IV}$ = 0.11 s, $1/k_V$ = 0.44 s, $1/k_{-I}$ = 0.01 s, $1/k_{-II}$ = 0.01 s, and $1/k_{-III}$ = 100 s.

When these values are entered into a computer spreadsheet which also contains the equations above to calculate the various parameters characterizing transcript initiation, one obtains τ_{o2} = 0.06 s, τ_{init} = 0.02 s, τ_{TC10} = 0.11 s, τ_{TC50} = 0.44 s, and τ_{min} = 0.56 s, corresponding to V_{max} = 106 initiations/minute; for K_m, a value of 4.35 μM is calculated, as observed. Further-

more, in glucose minimal medium, a relative value for $[R_f]$ was found to be 0.19 (Fig. 1a, second data point from the left), corresponding to 0.83 μM (= 0.19 × 4.35). With $[R_f]$ = 0.83 μM and the values for the eight rate constants as above, the spreadsheet calculates an initiation rate at the isolated *rrn* P2 of V = 17 transcripts/minute, as observed (Fig. 6c, triangles, second point from the left), and an average time required for promoter binding, τ_{c1} = 3.0 s, also as observed (Fig. 1c, second point from the right, value above the dotted line). It should be noted that the equations above and the spreadsheet based on them are applicable to any promoter under any conditions.

The assumed values for k_{II}, k_{III}, k_{-I}, and k_{-II} are lower limit values; these rates might be faster without much change in the promoter properties. For example, if these four rates were increased 10-fold, the promoter strength (V_{max}/K_m) would remain unchanged; also, the number of times the closed and open complexes have to be formed per initiation would remain unchanged.

The rate involved in promoter binding, k_I, is determined by the observed K_m value as long as the ratio k_{-I}/k_{II} is not changed. Furthermore, k_{-III} might be lower, i.e., the initiation complex might be stable for several minutes rather than for only 100 s, as assumed above, but again, this would have little effect on the promoter properties; i.e., the formation of the transcription complex is essentially irreversible, so that it is formed only once per initiation event. The reversion rates k_{-I} and k_{-II} could not be too much larger, because too many repeats of the open complex formation would reduce V_{max} below the observed value unless the forward rates are also assumed to be faster. If the reversion rates k_{-I} and k_{-II} are increased fivefold in comparison to the model above, so that $1/k_{-I}$ = 0.0002 s and $1/k_{-II}$ = 0.0002 s, then $1/k_I$ would have to be shortened to 0.022 s to obtain the observed K_m value, and closed and open complex formation would have to be repeated 111 and 11 times (rather than 7 and 3 times) per initiation event, but V_{max} and promoter strength would remain nearly unchanged. However, it is not clear whether such fast polymerase binding and the associated 111 repeats of the binding are compatible with actual diffusion rates within the cytoplasm, which set an upper limit to those rates.

Thus, the assumed values for the rate constants above give approximately correct V_{max} and K_m values and reflect the instabilities of the closed and open complexes and the stability of the initiation complex. These values are adjustable within broad limits without much change in the promoter properties and are consistent with observations. This set of values is considered a first attempt to model the process of *rrn* transcript initiation; this model can be expected to be refined in the future as more observational data become available.

Reduction of P1 promoter strength by ppGpp. In the absence of Fis, ppGpp reduces the strength of the *rrn* P1 promoter maximally about fivefold (Fig. 7d) (143). This might be entirely a direct effect of ppGpp binding to the RNA polymerase. However, as was mentioned above, it cannot be excluded that part of this reduction in promoter strength reflects the effect of a still-unidentified inhibitor that binds to the upstream region of the P1 promoters and whose synthesis is stimulated by ppGpp. In that case, the direct reduction in P1 promoter strength caused by ppGpp binding to the RNA polymerase would be less than fivefold.

For the lambda p_R promoter it was found that ppGpp inhibits the formation of the initiation complex or, more specifically, the formation of the first nucleotide bond of the emerging transcript (97). For this reason (see also reference 53), we assume that ppGpp reduces k_{III} for the *rrn* P1 promoter. If we assume an eightfold reduction in k_{III} and leave the other rate constants in the model above unchanged, we find that the promoter strength is reduced about fivefold, as observed in Fig. 7d. This is due to a fivefold increase in K_m from 4.35 to 24.4 and a modest 7% reduction in V_{max} from 106 to 99 initiations/minute. The effect of ppGpp might be amplified by a greater reversibility of open complex formation and promoter binding. For example, if we assume fivefold-increased reversibility with $1/k_{-I}$ = 0.0002 s and $1/k_{-II}$ = 0.0002 s discussed above, then a fivefold reduction in k_{III} would have sufficed to reduce the promoter strength about fivefold, again mainly by an increase in K_m.

From in vitro measurements of the time it takes to form the open complex at *rrnB* P1 at different ppGpp concentrations, Barker et al. (10, 11) recently concluded that ppGpp reduces the V_{max} of the P1 promoter (see Historical Overview above). However, the theoretical analysis presented here shows that in vivo V_{max} is essentially determined by the values for k_{IV} and k_V, which depend on the rRNA chain elongation rate. The rRNA chain elongation rate is independent of the growth rate (108, 134), presumably reflecting the combined effects of the antitermination sites of *rrn* operons and saturation with NTP substrates (see Historical Overview above). This makes it unlikely that, under in vivo conditions, ppGpp controls V_{max} at different growth rates.

PERSPECTIVE AND OUTLOOK

As was mentioned in the section above about the relationship between rRNA synthesis and growth rate, bacteria must balance the metabolic activities that go into either the production of ribosomes or the production of amino acids and factors for ribosome function in order to grow at an optimal rate with the given nutrients in the medium. Apparently, the mechanism to achieve this balance involves a control of the activity of ppGpp synthetase II, PSII (*spoT* gene product; see Introduction and Historical Overview above); whenever the ribosomes function at a submaximal rate, i.e., at a rate of less than 21 amino acid residues polymerized per second per average active ribosome at 37°C, PSII is activated, so that ppGpp accumulates and the synthesis of ribosomes is inhibited. As a result, the ribosome concentration, and therefore the consumption of amino acids by ribosomes, decreases until the metabolism is able to keep up the supply of amino acids for the reduced number of ribosomes. In this manner, the rate of ribosome function is somewhat reduced and the bacterial growth rate decreases to a lower level (see equation 2a above).

The available evidence suggests that the peptide chain elongation rate is monitored directly to activate PSII (Zhang et al., unpublished data). As a possible model, we have suggested that a low rate of translation of *spoT* mRNA favors a different folding of the SpoT polypeptide, so that it becomes the highly unstable ppGpp synthetase (40-s average life [89]) rather than the stable ppGpp hydrolase. In this manner, the synthesis of ribosomes could be adjusted to the capacity of the cellular metabolism to provide sufficient substrates for the ribosomes

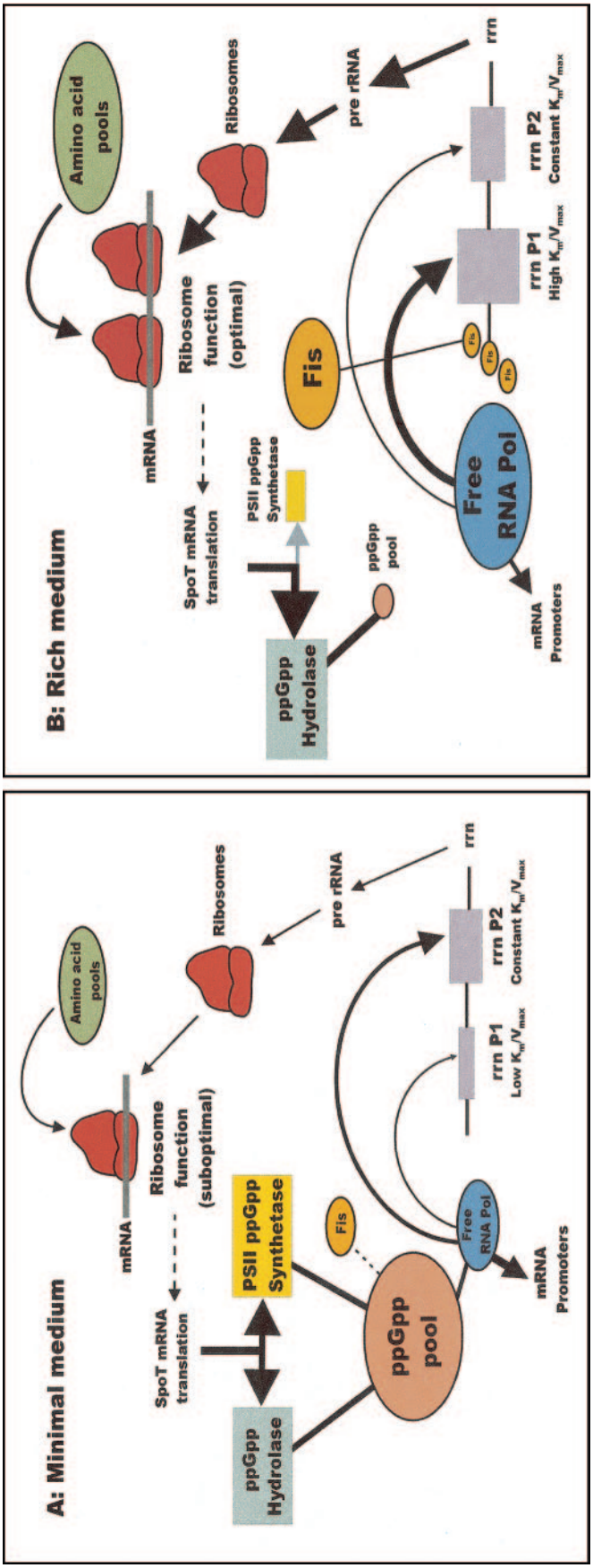

FIG. 10. Ribosome synthesis and growth rate. The states of the feedback loop that connects ribosome function and ribosome production to achieve an optimal growth rate in minimal (A) and rich (B) nutrient environments are illustrated. When the system is in balance, expanded growth and the rate of production of new ribosomes are tuned to the cellular capacity to increase the supply of amino acids and other substrates required to support protein synthesis. In the two examples, the concentrations of cellular components within the feedback circuit (amino acids, ppGpp, free RNA polymerase, and Fis) are indicated by the sizes of the ovals. The fluxes at different steps in the circuit (supply of amino acids, production of ppGpp, interaction of free RNA polymerase with the *rrn* P1 and P2 promoters, and ribosome biogenesis pathway) are indicated by the sizes of the arrows. During growth in minimal medium, where ribosome function is suboptimal, both ppGpp hydrolyase and ppGpp synthetase activities are produced from expression of the *spoT* gene, and ppGpp accumulates. During growth in rich medium, where ribosome function is optimal, only ppGpp hydrolyase is produced, and ppGpp does not accumulate. The relative strengths of the *rrn* P1 and P2 promoters (V_{max}/K_m) are illustrated by the sizes of the promoter rectangles. Important interactions are illustrated as solid lines connecting components. For example, during slow growth in nutritionally poor medium, the high concentration of ppGpp binds to the RNA polymerase and (i) inhibits the production of the *rrn* P1 transcriptional activator Fis and (ii) reduces the *rrn* P1 promoter strength (V_{max}/K_m). During rapid growth in rich medium, where the ppGpp concentration is low, Fis accumulates and activates the P1 promoter. For details, see the text. The ppGpp hydrolyase and PSII ppGpp synthetase are alternative activities encoded by the *spoT* gene.

to function efficiently. These ideas are schematically illustrated in Fig. 10.

During growth in minimal media with poor carbon sources, some of the basal level of ppGpp originates from RelA (106). However, this contributes little to the control of rRNA synthesis because under such conditions most *rrn* transcription comes from the P2 promoters (141), so that the growth rate-dependent control of rRNA synthesis is essentially the same in *relA*$^+$ and *relA* bacteria.

Any control that involves the generation of a signal that adjusts the controlled parameter, as opposed to a simple reaction equilibrium, can be referred to as feedback control (see section Ribosome Feedback Models above). Therefore, the control of rRNA synthesis as proposed above can be referred to as ribosome feedback. Several other ribosome feedback hypotheses have been proposed previously involving free ribosomes (60), translating ribosomes (26), or *rrn* gene dosage (114). Since neither free nor translating ribosomes accumulate to higher levels during slow growth in comparison to fast growth, the mechanism must have a more complex response pattern than envisioned initially. The new ribosome feedback proposed here differs from the previous hypotheses in that it assumes that the velocity of translation by ribosomes, not the concentration of free or translating ribosomes, is the controlled parameter, deviations from which generate the controlling signal ppGpp.

With a role for ppGpp in the growth medium-dependent control of rRNA synthesis now well supported, the question is what remains for the future? One of the most important problems with respect to the control of ribosome synthesis and growth is to establish the mechanism of the growth medium-dependent control of ppGpp accumulation that involves the enzymatic ppGpp synthetase and ppGpp hydrolase activities of the *spoT* gene. Does this control indeed involve *spoT* mRNA translation as proposed above, or is SpoT a bifunctional enzyme whose activity is controlled by metabolic signals related to ribosome function? In addition, several more complex questions remain unanswered. One of these returns us to the balance between ribosome synthesis and function. According to equation 2a above, the same rate of protein synthesis and growth rate (i.e., where growth rate is equivalent to protein production) can be achieved with different pairs of values for ribosome synthesis (i.e., number of ribosomes per amount of protein) and function (i.e., the rate of peptide chain elongation) as long as the product of these parameters remains unchanged. The question is, what determines the fine tuning of the particular set of values for these parameters? Only if we understand how these parameters remain in metabolic balance under a given condition can we say that the problem of the control of ribosome synthesis and bacterial growth is solved.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health, the Swedish Research Council, the Canadian Institute for Health Research, and the National Science Foundation.

The opinions, findings, and conclusions expressed in this publications are those of the authors and do not necessarily reflect the views of the National Science Foundation.

ADDENDUM

We acknowledge two relevant papers published after submission of this review. The first paper describes a protein factor, DksA, that binds to RNA polymerase and participates in the control of the *rrn* P1 promoter by ppGpp (B. J. Paul, M. M. Barker, W. Ross, D. A. Schneider, C. Webb, J. W. Foster, and R. L. Gourse, Cell **118:**311–322, 2004). In a *dksA* deletion strain, the stringent response to amino acid starvation is nearly abolished and, during slow growth, the *rrn* P1 promoter expression and the stable RNA-to-protein ratio are greatly increased in comparison to a wild-type strain. Furthermore, the inhibition of in vitro transcription from *rrn* P1 by ppGpp is increased tenfold by the addition of DksA. Thus, DksA appears to be required for the inhibition of *rrn* P1 activity by ppGpp. Although the mechanism of ppGpp action is not a topic in our review, it is noteworthy that these findings are important and fully compatible with our conclusion that ppGpp is the sole factor responsible for the control of rRNA synthesis during exponential growth at different rates. The reason is that the DksA levels were found to remain unchanged with growth rate and growth phase and thus do not confer a novel type of regulation, independent of ppGpp concentration.

We also note that the interpretation of the data from P1 expression and the RNA/protein ratio (*R*/*P*) is more complex than assumed by the authors. In wild-type bacteria growing in rich, glucose-amino acids medium, *R*/*P* equals 6 (μg/μg), and the absence of DksA does not change this ratio. In contrast, in glucose minimal medium, the absence of DksA increases *R*/*P* nearly twofold, from 3.5 to 6 (μg/μg). This seems to suggest that the absence of DksA increases the rate of rRNA synthesis during growth in minimal media, when ppGpp levels are high, but not in rich media when ppGpp levels are close to zero. Although intuitively plausible, this interpretation may not be correct. Based on the definition of exponential growth (equation 1 above), *R*/*P*, together with the growth rate, determines the rate of rRNA synthesis per amount of protein and the rate of protein synthesis per ribosome ("ribosome efficiency"). The calculation shows that the absence of DksA reduces the rRNA synthesis rate per amount of protein by 33% in glucose-amino acids medium and by 15% in glucose minimal medium, in contrast to the intuitive appearance. In addition, the absence of DksA reduces the ribosome efficiency in the two media by 30 and 15% and the growth rate by 33 and 50%, respectively. These considerations highlight the complexities in interpreting the effects of DksA on the bacterial physiology; clearly, further analysis will be required for a complete understanding.

In another paper (H. D. Murray and R. L. Gourse, Mol. Microbiol. **52:**1375-1387, 2004), the authors reiterate earlier conclusions (87) that the *rrn* P2 promoter is regulated by ppGpp and iNTP's. In vitro transcription from *rrn* P2 was 50% inhibited by ppGpp; but no inhibition occurred with a mutant promoter P2(dis). In addition, relative amounts of P2 reporter transcripts and of NTPs were measured in vivo after transitions from stationary to exponential phase and vice versa, and after nutritional upshifts. The in vivo experiments are difficult for us to interpret, since neither the absolute P2 promoter activities, nor the cytoplasmic NTP concentrations were determined; i.e., none of the changes associated with the shifts that alter cell volumes (for cytoplasmic NTP concentrations), DNA replica-

tion (to determine activities per promoter), reporter transcript turnover, and free RNA polymerase were measured and taken into consideration. In vitro, ppGpp decreases the formation and stability of the open complex, but since open complex formation at *rrn* promoters is more than 100 times faster in vivo than in vitro and therefore not limiting the rate of initiation, ppGpp is not expected to measurably affect the P2 activity in vivo. In fact, previous results showed no significant differences in the growth rate-dependent regulation of the absolute activity of *rrn* P2 promoters in the presence or absence of ppGpp (78). Thus, the new in vitro results do not invalidate previous in vivo observations that showed the activity of the isolated *rrn* P2 promoter to be unaffected by ppGpp during exponential growth at different rates.

Finally, we acknowledge a publication by L. Jöres and R. Wagner (J. Biol. Chem. **278:**16834-16843, 2003) that suggests that essential steps in the ppGpp-dependent regulation of bacterial rRNA promoters can be explained by substrate competition. Those results support our conclusion that ppGpp reduces the rate constant k_{III} for the formation of the initiation complex (see "Mathematical modeling of the control of *rrn* transcript initiation" above). This is in contrast to other proposals that ppGpp exerts its effect by reducing k_{II}, the rate of open complex formation (10, 11).

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